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MOLECULAR BIOLOGY & RECOMBINANT DNA TECHNOLOGY

Dr. William S. Reznikoff

Dr. Gary Buell

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AMERICAN CHEMICAL SOCIETY SHORT COURSES

Course: MOLECULAR BIOLOGY AND RECOMBINANT DNA TECHNOLOGY

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Location: Washington, DC Beginning Date: August 24, 1990

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MOLECULAR BIOLOGY & RECOMBINANT DNA TECHNOLOGY

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Dr. William S. Reznikoff

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8:30 Sat AM

Molecular Biology and Recombinant DNA

A. Introduction

1) Biochemical Genetics; Genes Function by Encoding Proteins

B. Molecular Biology

1) Structure of DNA and Nucleic Acid Hybridization Studies
2) Transcription; RNA Synthesis is the First Event in Gene Expression, the Initiation Step is Crucial
3) Translation; the mRNA to Protein Decoding Process
4) Special Aspects of Gene Expression; Eukaryotic mRNA Processing; Protein Modification
5) Chromosome Duplication; the Enzymology of DNA Synthesis, Origins of DNA Replication

C. Tools and Techniques used in Manipulating DNA Molecules

1) DNA Polymerase
   Reverse Transcriptase
   Terminal Transferase
   Phosphatase and Kinase
   Nucleases
   Restriction Enzymes
   Ligase
2) Gel Electrophoretic Separation of Polynucleotides
3) DNA Sequence Analysis
4) Polymerase Chain Reaction (PCR) Technology

D. Cloning -- Forming Recombinant DNA Molecules

1) Outline of "Typical Protocol"
2) Choice of Vehicle or Vector; Relationship to Host, Detection in Host, Nature of Product DNA
3) Cloned DNA; Genomic, Copy or Synthetic DNA (Source of Foreign DNA)
4) Joining Vector and Cloned DNA; Cohesive Ends, Blunt Ends, Tails, Linkers
5) Detecting Successful Clones; Loss of Function (Insertion into Vector Gene), Phosphatase Vector Ends, Gain of Function, Nucleic Acid Hybridization Analysis, DNA Sequence Analysis

E. <u>In Vitro</u> Mutagenesis

1) Cloning Generates Insertions and Deletions
2) Nested Deletion Formation
3) Localized Chemical Mutagenesis
4) Synthetic Oligonucleotide Directed Mutagenesis
5) Synthetic Genes
6) PCR and DNA Manipulation

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F. Expression Foreign Genes

1) Vectors with Appropriately Placed Expression Signals; Promoter, Translation Initiation Signal
2) Trouble Shooting Failures; "Unstable" Recombinant DNA, Expression is Deleterious to Host, Stabilizing Product Protein, Translation Initiation Problems, mRNA Instability, Codon Usage
3) Using Fusions to Study Foreign Gene Expression

G. Polynucleotide Probes

1) Identification of Organisms
2) Southern Blots and Restriction Fragment Polymorphisms
3) Detecting Single Base Pair Changes with Oligonucleotide Probe Hybridization

H. Example case of protein overproduction

I. E. coli to produce monoclonals

J. cloning & Production of Interferons

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REFERENCES

ACS Course in Molecular Biology and Recombinant DNA Technology

General References:

Molecular Biology of the Gene by Watson, Hopkins, Roberts, Steitz and Weiner. Benjamin/Cummings Publishing 1987

Recombinant DNA: A Short Course by Watson, Tooze, and Kurtz. W.H. Freeman and Co. - Scientific American Books 1983

Remember that Watson is dogmatic – truth may not be as clear-cut

Techniques References:

Current Protocals in Molecular Biology by Ausubel, Brent, Kingston, Moore, Seidman, Smith, and Struhl. John Wiley and Sons Publishers 1987
(annual) updates are sent periodically

Molecular Cloning: A Laboratory Manual by Maniatis, Fritsch, and Sambrook. Cold Spring Harbor Laboratory ~~1982~~ no other versions available

From Genes to Clones: Introduction to Gene Technology by Winnacker. VCH Verlagsgesellschaft or VCH Publishers 1987

Laboratory courses also 3:
1. ACS
2. Various courses
3. Cold Spring Harbor; CV, etc.

Journals: Science, Nature
Nucleic Acids Research
Gene
PNAS
J. Bacteriology
J Biol Chem

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GLOSSARY

ACS Course in Molecular Biology and Recombinant DNA Technology

1. anneal - To form double stranded polynucleotides from two species of complementary single stranded polynucleotides.

2. bank - also called library - A large collection of recombinant DNA molecules derived from a single tissue or cell type.

3. blunt end - The end of a double stranded DNA molecule in which neither strand is protruding. A synonym is "flushed end".

4. CAP - The modified 5' end of eukaryotic mRNA's.

5. cDNA - complementary or copy DNA - A DNA copy of an RNA molecule which is generated in vitro with reverse transcriptase.

6. clone - A specific recombinant DNA molecule (either in its purified state or within host organism or inside a viral capsid). An alternative meaning not used in this course refers to an organism which is genetically identical to another -- usually the one from which it was derived. To clone -- the generation of recombinant DNA molecules.

7. code - The 64 possible codons all but three of which encode amino acids during protein synthesis.

8. codon - A 3 base RNA sequence which encodes a given amino acid during protein synthesis (translation).

9. cohesive ends - Short single stranded extension of double stranded DNA molecules which are complementary to each other. A synonym is "sticky ends".

10. complementarity - A measure of the extent to which two single stranded polynucleotides can form a base-paired double stranded structure. Sometimes the word "homology" is used as a synonym.

11. continuous strand - The newly made DNA strand which is synthesized in a continuous fashion because its overall orientation is 5' to 3'. A synonym is "leading strand".

12. degeneracy - A property of the code in that a given amino acid is, in most cases, encoded by more than one codon.

13. denaturation - Treating a macromolecule so that it looses its native structure. Usually this is accomplished without the breaking of any covalent bonds. Denaturation of double stranded DNA molecules involves the separation of the two complementarity single stranded molecules.

14. discontinuous strand - The newly made DNA strand which is synthesized in a discontinuous fashion; in short segments a few thousand bases in length. This occurs because its overall orientation (3' to 5') is the opposite of that which DNA polymerases are capable of synthesizing. A synonym is "lagging strand".

15. duplex - Double stranded DNA molecule.

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16. exon - Portion of a genomic DNA sequence which encodes a portion of a mature mRNA. Many eukaryotic genes are composed of two or more exons separated by introns whose corresponding RNA sequences are removed (spliced out) during maturation.

17. fidelity - The fraction of correctly base paired nucleotides in a duplex.

18. flush end - Blunt end.

19. gene - A DNA sequence shich encodes a protein. A cistron is an equivalent name.

20. genomic DNA - DNA derived from chromosomes.

21. homology - Complementarity.

22. host - The organism (usually single celled) into which the recombinant DNA molecule is introduced and propagated.

23. hybridization - The formation of a double stranded polynucleotide molecule when two complementary strands are brought together under appropriate conditions. These strands can be either DNA or RNA or one of each.

24. hyperchromic shift experiment - An analysis of double stranded polynucleotide denaturation by following the increase in optical density at 260 nm.

25. initiation codon - Usually AUG; the codon which can correspond to formyl methionine. Formyl methionine is the first N terminal amino acid initiating the synthesis of all proteins.

26. intron - The portion of genomic DNA which does not encode a mature RNA sequence (see exon).

27. lacZ - The gene in E. coli which encodes the enzyme ß-galactosidase.

28. lagging strand - Discontinuous strand.

29. leading strand - Continuous strand.

30. library - A collection of random recombinant DNA molecules (either as free DNA or encapsulated as virious or in the host cells) which because of the average size of the cloned DNA, the size of the genome from which they were derived and the number of different molecules, are likely to represent a certain percentage of the entire genome.

31. ligation - The bringing together of two double stranded molecules through the formation of phosphodiester bonds.

32. linker - A small synthetic double stranded DNA molecule. Usually these contain sequences recognized by some restriction endonucleases.

33. mRNA - messenger RNA - An RNA molecule which is translated by ribosomes into 1 or several proteins.

34. melting - Denaturation; usually accomplished by heating a solution of double stranded polynucleotides.

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35. Northern blot - A hybridization technique for detecting the location of specific RNA molecules which have been fractionated by P.A.G.E.

36. oligo (oligonucleotide) - A short RNA or DNA strand; typically less than 30 bases in length.

37. operon - A group of linked genes which function together as a group. Operons exist in prokaryotes but probably not in eukaryotes. An operator is a sequence in the DNA at which regulation of operon gene expression occurs.

38. P.A.G.E. - Polyacrylamide gel electrophoresis - A technique which separates various species of proteins or polynucleotides in an electrical field.

39. phage - A virus which attacks bacteria.

40. plasmid - A secondary circular DNA molecule which is found in a cell. Usually it encodes "dispensible functions", is smaller than the main chromosome(s), and may be found in multiple copies per cell.

41. polarity - The chemical orientation of a single stranded polynucleotide. The two strands of a duplex are antipolar (in reverse polarity).

42. poly-A sequence - The poly A sequence added to the 3' end of most eukaryotic mRNAs after their synthesis.

43. primer - An oligo or polynucleotide complementary to a template single strand with a free 3'-OH to which deoxyribonucleotides are added during DNA synthesis.

44. probe - A radioactively labeled oligonucleotide used to detect complementary sequences in a hybridization experiment.

45. promoter - A DNA sequence to which RNA polymerase binds during the transcription initiation reaction.

46. reading frame - The "correct" frame of reference in "reading" codons in an mRNA during translation.

47. recombinant DNA - A DNA molecule in which the DNA sequence of interest has been attracted to a vector or vehicle DNA such that it can be replicated in the host of choice.

48. redundancy - Degeneracy.

49. relaxed conditions - Hybridization conditions which allow short ($\leq$ 20) duplexes to form between molecules which cannot base pair at each position.

50. renaturation - To reform duplex DNA molecules from a denatured mixture.

51. replication - The copying of a DNA molecule yielding two DNA molecules identical to the original.

52. repressor - A protein which regulates a specific operon by binding to its operator.

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53. restriction digest - The products of a restriction endonuclease cleavage of a DNA sample.

54. Shine-Dalgarno sequence - A sequence in prokaryotic mRNA's which proceeds the translation initiation codon AUG and which participates in base pairing with the 16S ribosomal RNA.

55. shotgun cloning - The formation of recombinant DNA molecules from a random assortmant of target DNA molecules.

56. Southern blots - A hybridization technique for detecting the location of specific DNA sequences which have been fractionated by P.A.G.E. The DNA molecules have usually been cleaved by a restriction endonuclease prior to P.A.G.E.

57. splicing - The step in eukaryotic mRNA maturation in which introns are removed and exons are linked together.

58. stringent conditions - Hybridization conditions which allow short ($\leq$ 20) duplexes to form only when base pairs form at all positions (high temperatures, low salt).

59. synthetic DNA - A chemically synthesized DNA sequence.

60. tail (homopolymer) - A run of usually identical bases which is added to the 3' ends of DNA molecules in some cloning experiments.

61. template - An RNA or DNA single strand which determines the nucleotide to be added during DNA or RNA synthesis through base pairing rules.

62. termination codon - Codons which do not encode an amino acid; usually UAA, UAG and UGA. Also called stop codon.

63. 3' end processing - The maturation of the 3' end of eukaryotic mRNA's. This includes cleavage and poly A addition.

64. Tm - The temperature at which apparently 50T of the DNA denaturation has occurred for a given type of DNA in a thermal denaturation experiment. The Tm is a measure of the stability the base pairing of a duplex.

65. transcription - The synthesis of an RNA molecule from a DNA template (reverse transcription is the synthesis of a DNA molecule from an RNA template).

66. translation - The synthesis of a protein molecule which is programmed by an mRNA.

67. triplet - The three RNA bases in a codon.

68. vector - The "carrier" DNA molecule used in recombinant DNA experiments. When E. coli is used as a host organism this is typically derived from a plasmid or a phage DNA molecule.

69. vehicle - Vector.

70. Western blot - An immunological technique for detecting proteins which have been fractionated by P.A.G.E.

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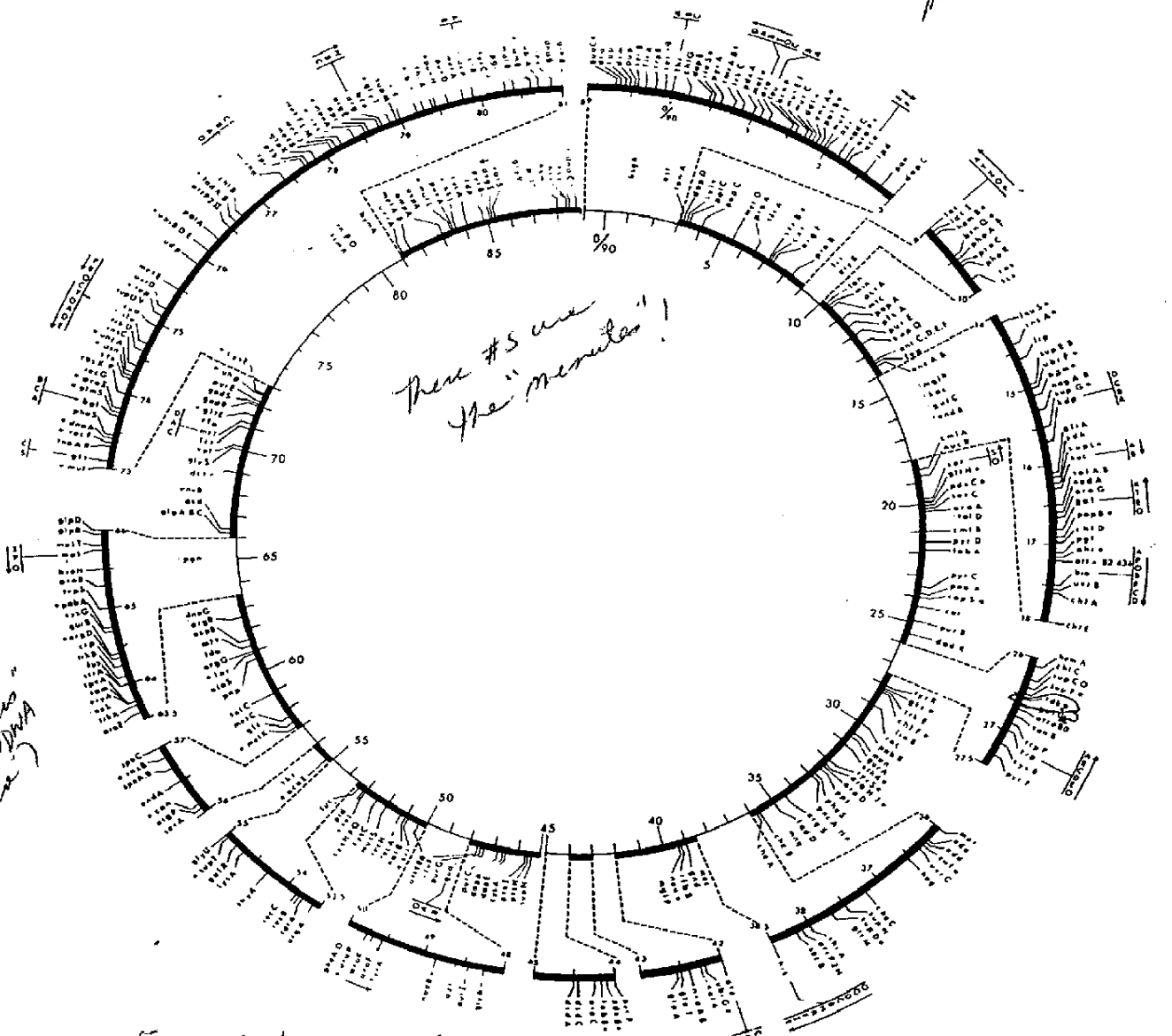
4000 genes in E. coli — 25% done
gene ≡ (in most cases) a stretch of DNA that encodes a protein
phenotype = properties

FIG 1 - A GENETICIST'S VIEW OF THE E. COLI CHROMOSOME.

→ representation from a pemalistic pt. of view

two circles 'cause not enough space

Linkage Map of *Escherichia Coli* K-12



these #s are the "minutes"!

guess is 1-2% "meaningless" (useless) DNA (consensus)

E coli has 4×10^6 bp
good seq lab can do 10×10^3 bp/day ⇒ 3 yrs for E. coli
human genome ~ 4×10^9 bp ⇒ one lab = 3000 years
$ = 204 → $5 per bp

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(rbs) = ?

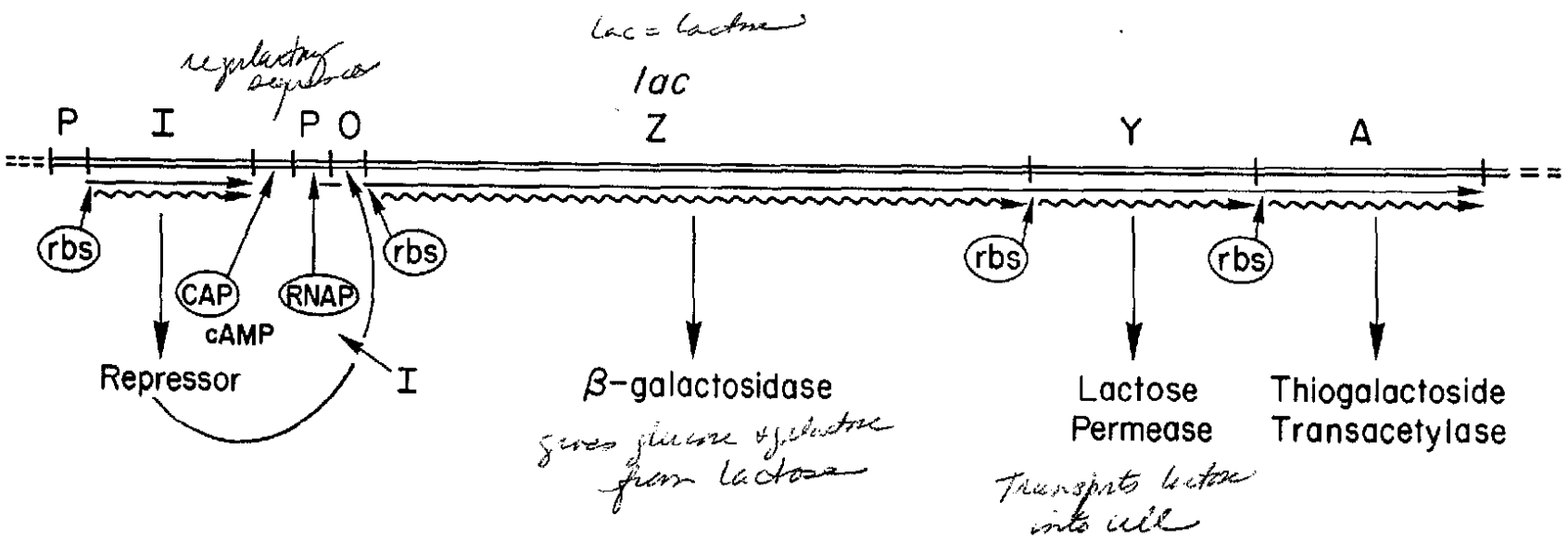

FIG 2 - THE LACTOSE (LAC) OPERON GENES. THE LAC OPERON IS COMPOSED OF THREE GENES WHICH ENCODE THE ENZYMES (PROTEINS) BETA-GALACTOSIDASE, LACTOSE PERMEASE AND THIOGALACTOSIDE TRANSACETYLASE. A NEARBYE GENE ENCLODES A REGULATORY PROTEIN. THE OPERON ALSO CONTAINS SOME DNA SEQUENCES WHICH CORRESPOND TO GENETIC START, STOP AND REGULATORY SIGNALS.

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FIG 3 - THE END PRODUCT OF GENE EXPRESSION IS THE SYNTHESIS OF PROTEINS. THE BUILDING BLOCKS OF PROTEINS ARE 20 AMINO ACIDS.

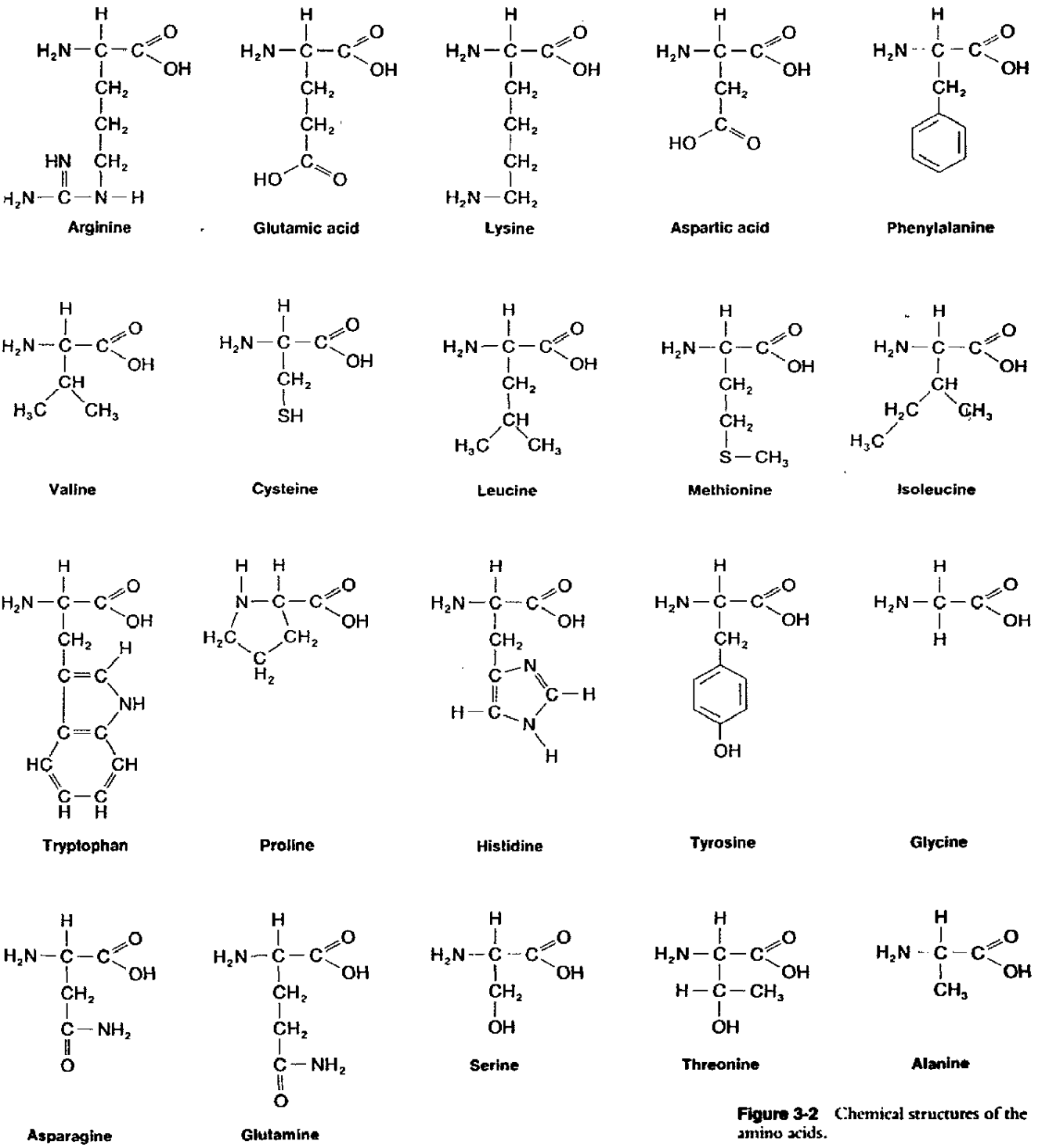

Figure 3-2 Chemical structures of the amino acids.

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FIG 4 - THE AMINO ACIDS ARE LINKED TOGETHER THROUGH PEPTIDE BONDS. RUNS OF AMINO ACIDS CAN FORM 2^{O} STRUCTURES SUCH AS ALPHA HELIXES.

Figure 3-3 Formation of a dipeptide from two amino acids by elimination of water (shaded circle) to form a peptide group (shaded rectangle).

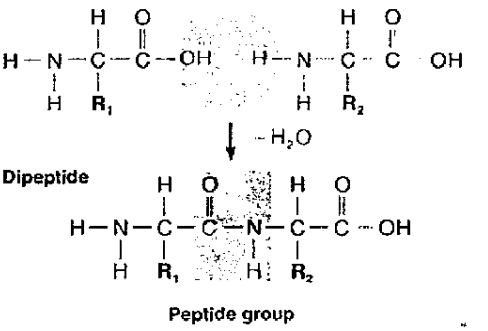
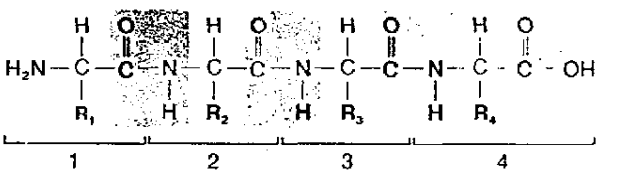


Figure 3-4 A tetrapeptide showing the alternation of α-carbon atoms (red) and peptide groups (shaded). The four amino acids are numbered below.

amem - αC - CO - amino - αC - CO, etc



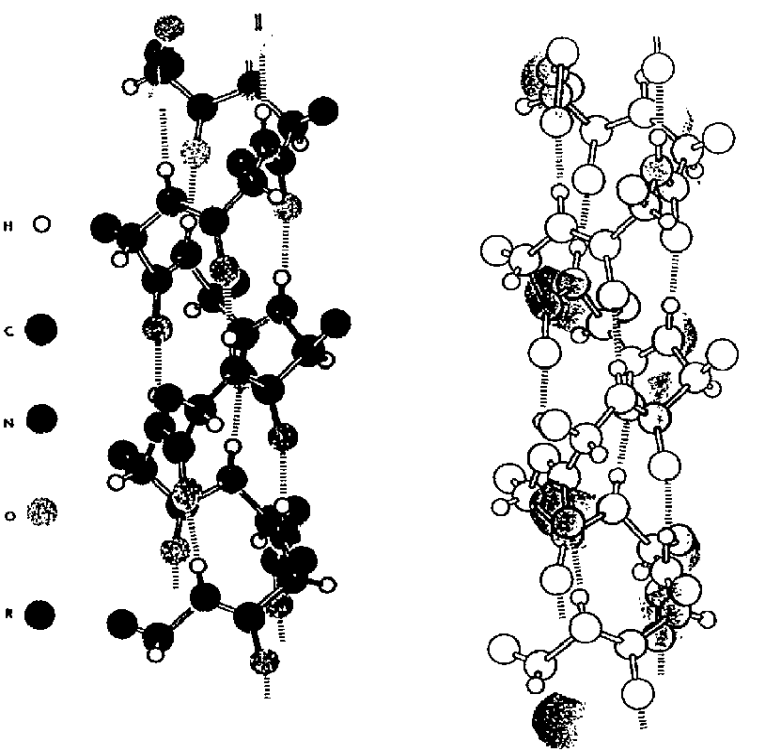


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FIG 5 - GENES ENCODE THE PRIMARY STRUCTURE (THE ORDER OF AMINO ACIDS) OF PROTEINS. THE 1^{O} STRUCTURE OF A PROTEIN IN TURN DETERMINES THE 3° STRUCTURE OF THE PROTEIN BECAUSE OF THE INTERACTIONS OF THE VARIOUS AMINO ACID SIDE GROUPS WITH EACH OTHER AND WITH THE ENVIRONMENT. THE 3° STRUCTURE IS THE OVERALL CONFIGURATION OF THE PROTEIN. BY BRINGING TOGETHER VARIOUS AMINO ACID SIDE CHAINS IN PRECISE ARRANGEMENTS, THE TERTIARY STRUCTURE ENDOWS SOME PROTEINS WITH CATALYTIC PROPERTIES THUS CREATING ENZYMES.

dark = active site amino acids

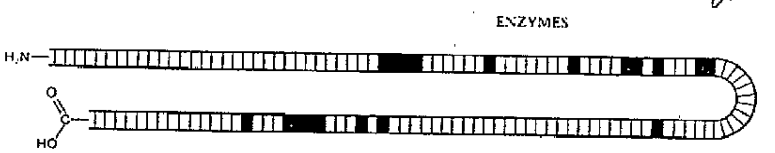

Figure 6-22 Schematic diagram of the amino acid sequence of lysozyme showing that the amino acids (red) in the active site are separated along the chain. Folding of the chain brings these amino acids together.

widely separated clusters along the chain. Only when the chain is folded do they come into proximity and form the active site. The folding of the chain is shown in Figure 6-23. The deep cleft indicated by the arrow is the active site. This is seen more clearly in the space-filling model shown in Figure 6-24. The substrate is a hexasaccharide segment that fits into the cleft and is distorted upon binding. The enzyme itself changes shape when the substrate is bound. A variety of interactions (van der Waals, hydrogen, and ionic bonds) stabilize the binding. Another enzyme, yeast hexokinase A, has been studied in order to examine what structural changes occur on substrate bind-

"silent mutation"

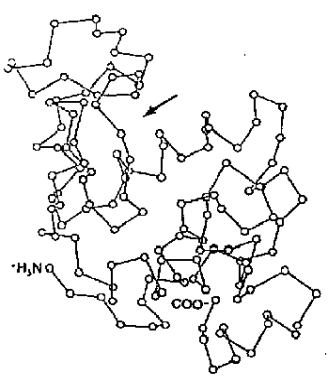

Figure 6-23 Three-dimensional structure of lysozyme. Only the α-carbon atoms are shown. The active site is in the cleft indicated by the arrow. [Courtesy of Dr. David Phillips.]

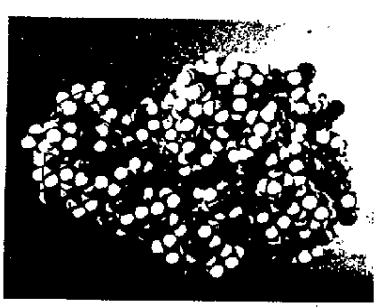

Figure 6-24 A space-filling molecular model of the enzyme lysozyme. The arrow points to the cleft that accepts the polysaccharide substrate. (C atoms are black; H, white; N, gray; O, gray with slots.) [Courtesy of John A. Rupley.]

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FIG 6A- THE E. COLI CHROMOSOME IS EXTREMELY COMPLEX; IT IS COMPOSED OF ABOUT 3,000 GENES OR 3×10^6 BASE PAIRS OF DNA.

circle has some superstructure

may be protein + RNA in core

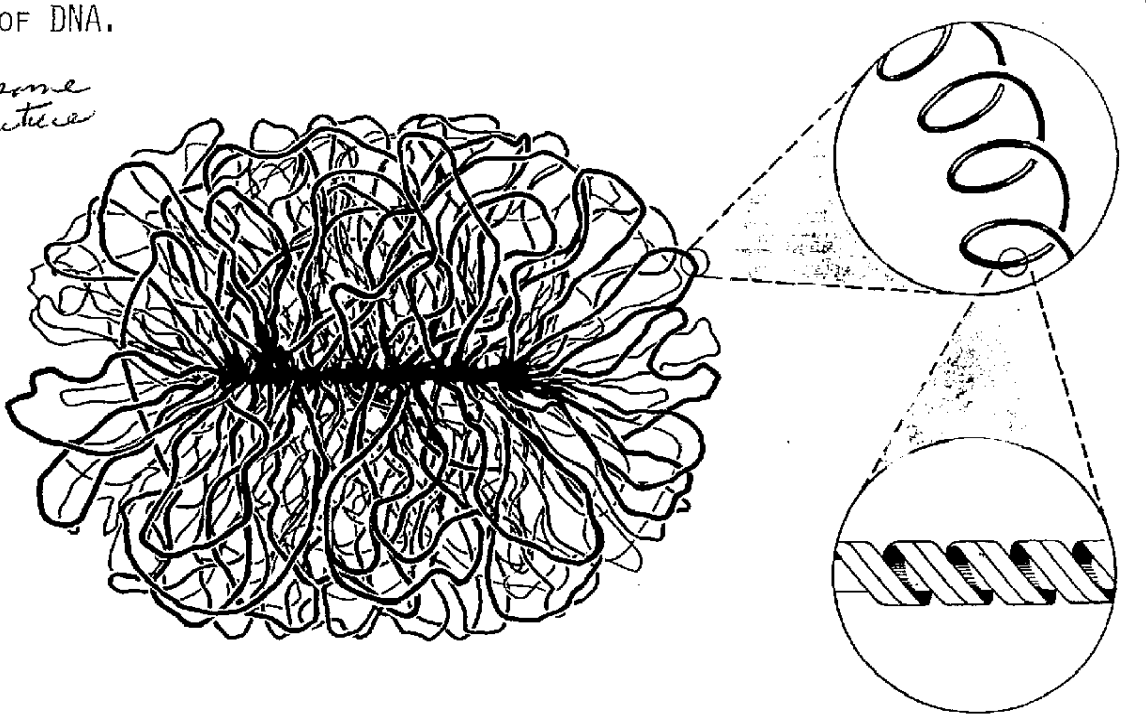
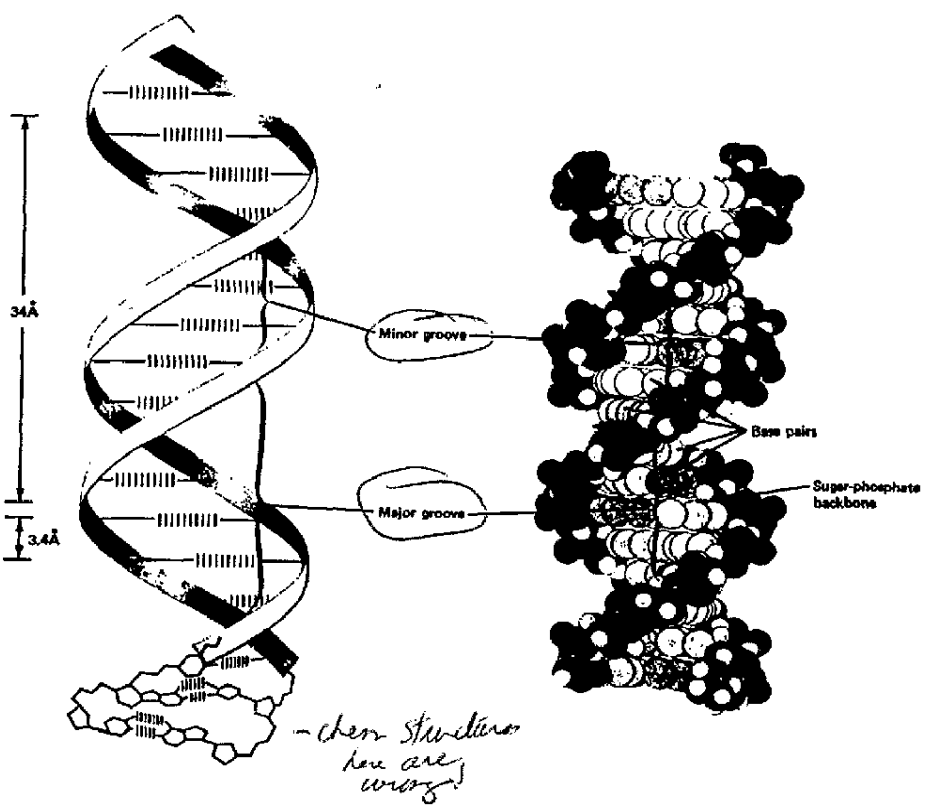


FIG 6B- A SCHEMATIC OF A SMALL SECTION OF DNA.



- chem structures here are wrong!

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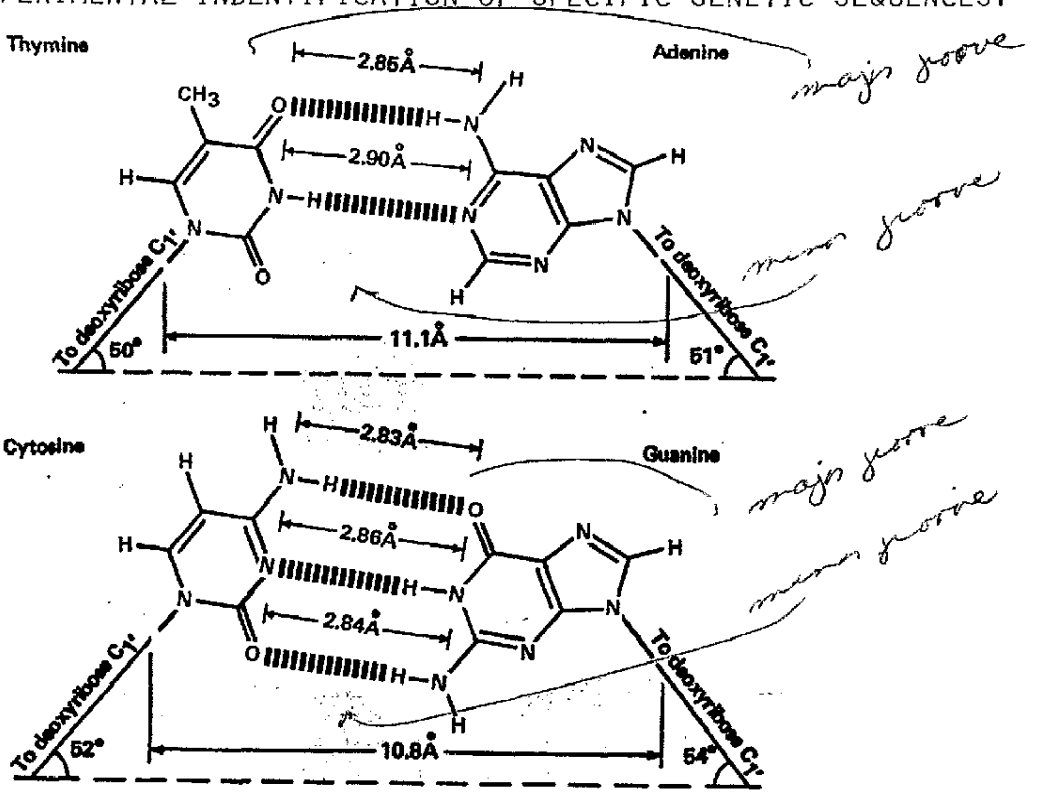
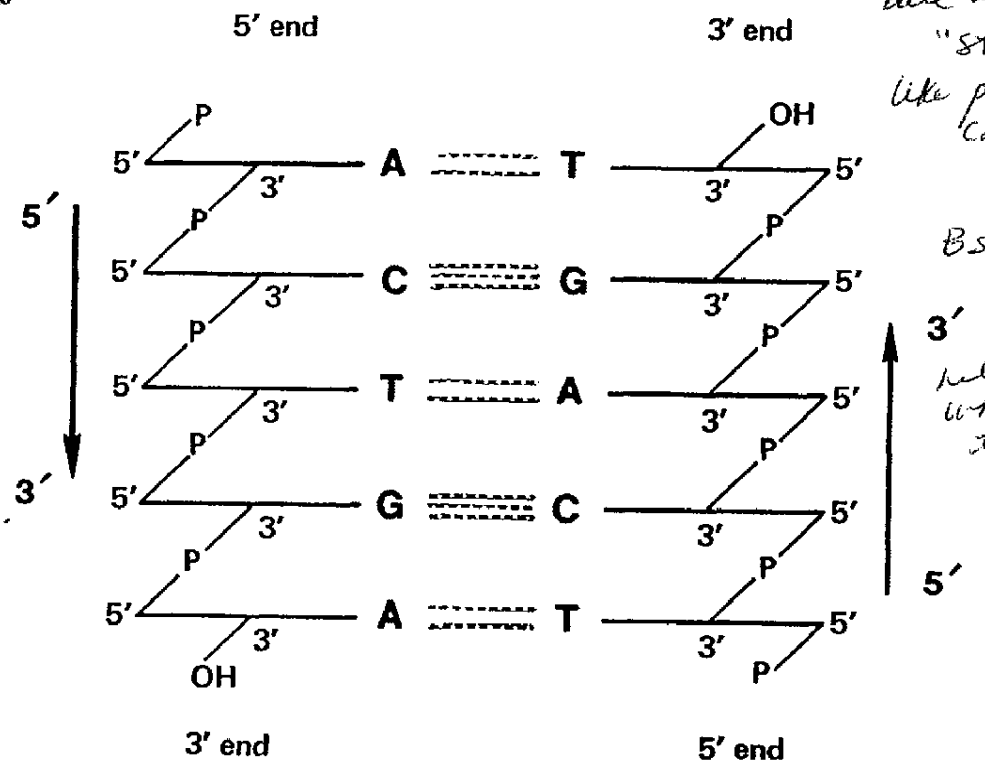
FIG 7 - BASE PAIRING OF DNA STRANDS (OR OF DNA::RNA DUPLEXES) IS THE MECHANISM OF INFORMATION TRANSFER AND IS IMPORTANT IN THE EXPERIMENTAL INDENTIFICATION OF SPECIFIC GENETIC SEQUENCES.





TA
CG

Chemistry could occur to differentiate the major grooves from each other

polynucleotide

bases also involved in "stacking" like pancakes — contributes to thermodynamic stability

B structure → planar

3′ I other helical structure which demonstrate stacking

5′

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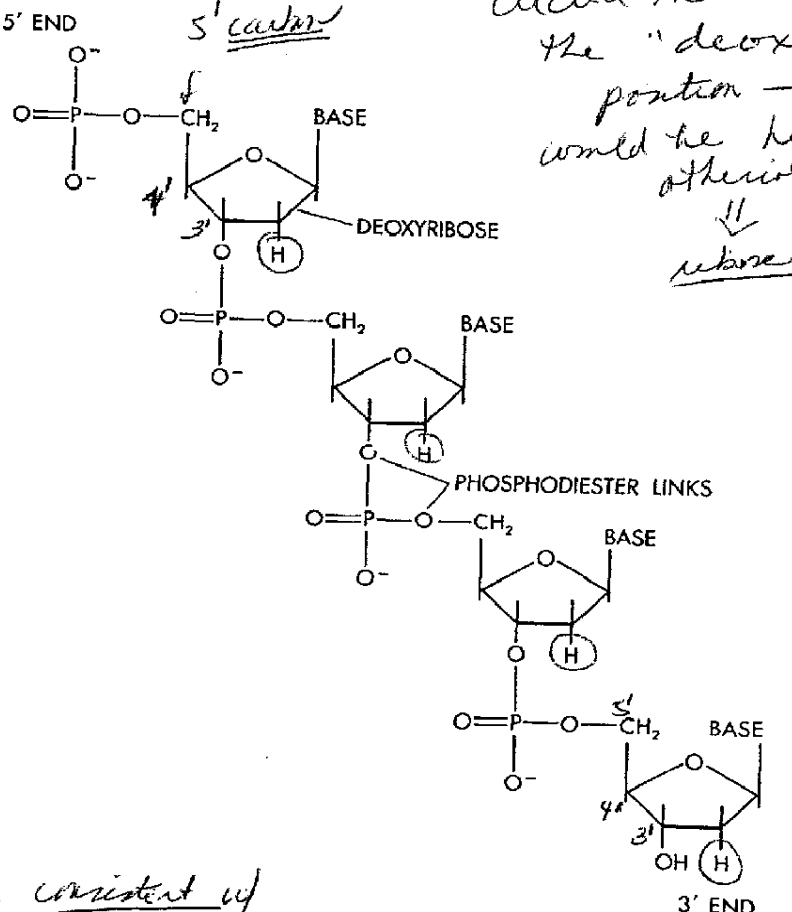
FIG 8 - THE PHOSPHODIESTER BACKBONE OF DNA.

5' ⇒ $PO_4^{\equiv}$
3' ⇒ sugar = hydroxyl

||
~~[illegible]~~
on outside

ante || backbones



circled Hs are the "deoxy" position — would be hydroxyl otherwise
⇓
ribose

note how phosphates link the sugars
condensation of $PO_4^{\equiv}$ leakages is how sugar (of backbone?) occurs

As a convention and consistent w/ biosynthesis, DNA sequences written 5' → 3'

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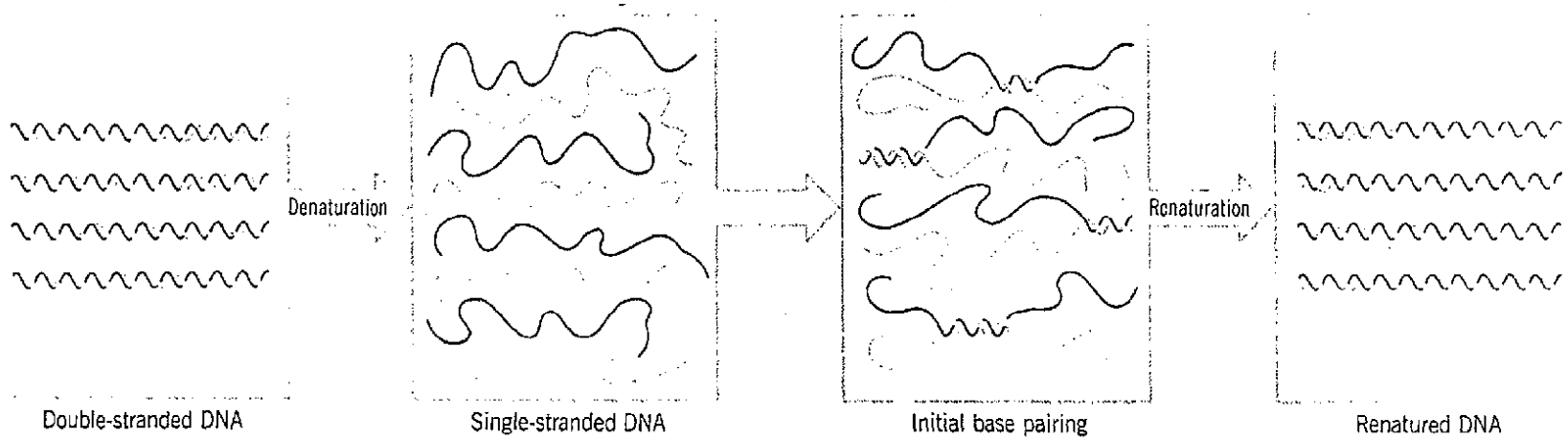
FIG 9- BASE PAIRING IS STUDIED BY DENATURATION-RENATURATION EXPERIMENTS. THE TEMPERATURE REQUIRED TO DENATURE (MELT) DNA IS ABOUT 25° HIGHER THAN THE OPTIMAL TEMPERATURE FOR RENATURATION.

to denature: Δ pH (11 will do)
heat
hydrophobic solvents

Denatured single strands of DNA can renature to the duplex form.

relevant [ ] same here



Max rate of renaturation occurs at (T_M − 25°) degrees

Denaturation – breaking strands apart
Melting = denaturation when applied to DNA
= thermal denaturation

Renaturation – backwards process – but 2 strands are not necessarily both from same original source
Hybridization – syn to renaturation, but ⇒s 2 molecules are from different sources
Anneal – More general; covers both possibilities

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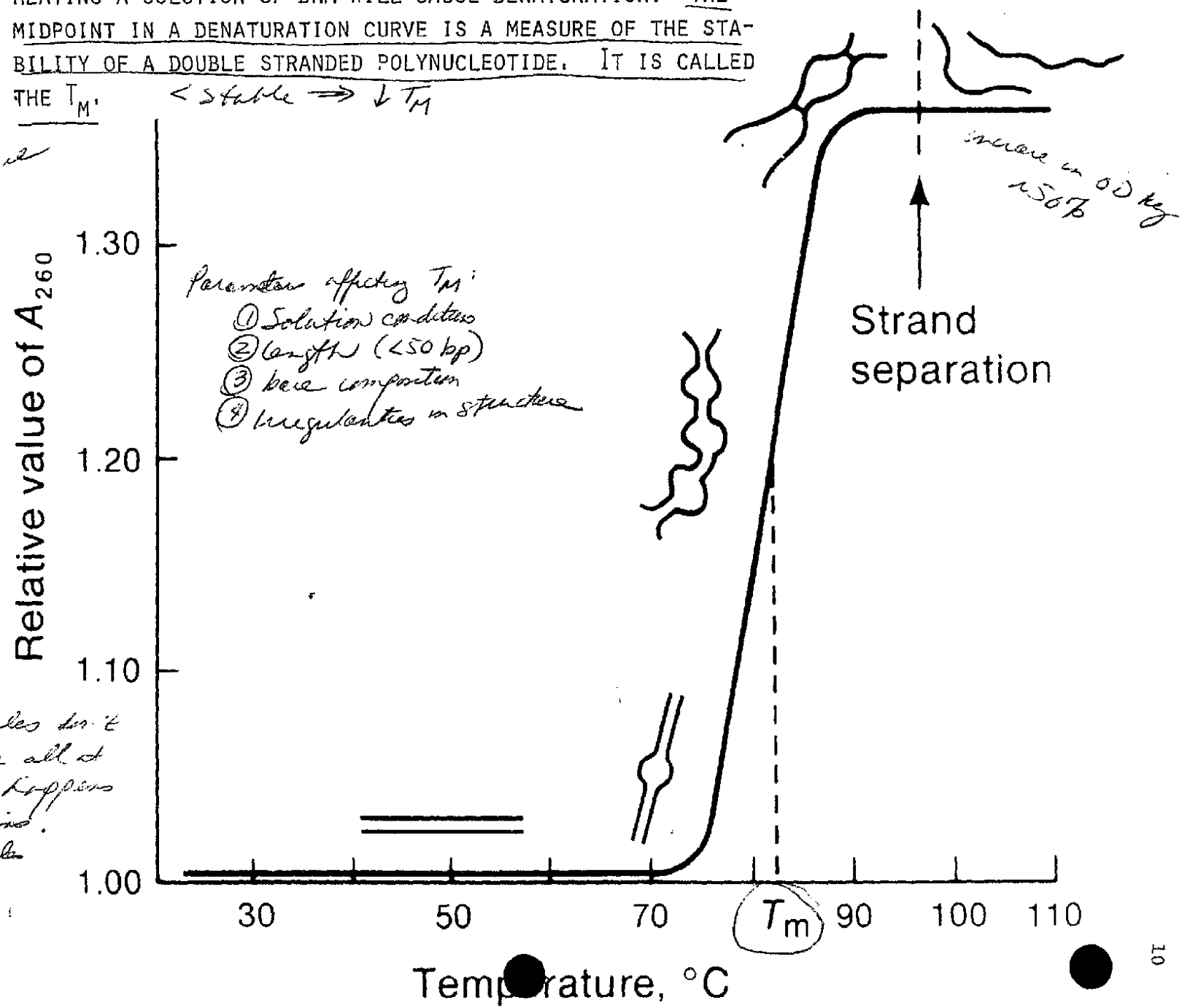
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 10- DNA DENATURATION INVOLVES SEPARATION OF THE TWO STRANDS. HEATING A SOLUTION OF DNA WILL CAUSE DENATURATION. THE MIDPOINT IN A DENATURATION CURVE IS A MEASURE OF THE STABILITY OF A DOUBLE STRANDED POLYNUCLEOTIDE. IT IS CALLED THE T_M.



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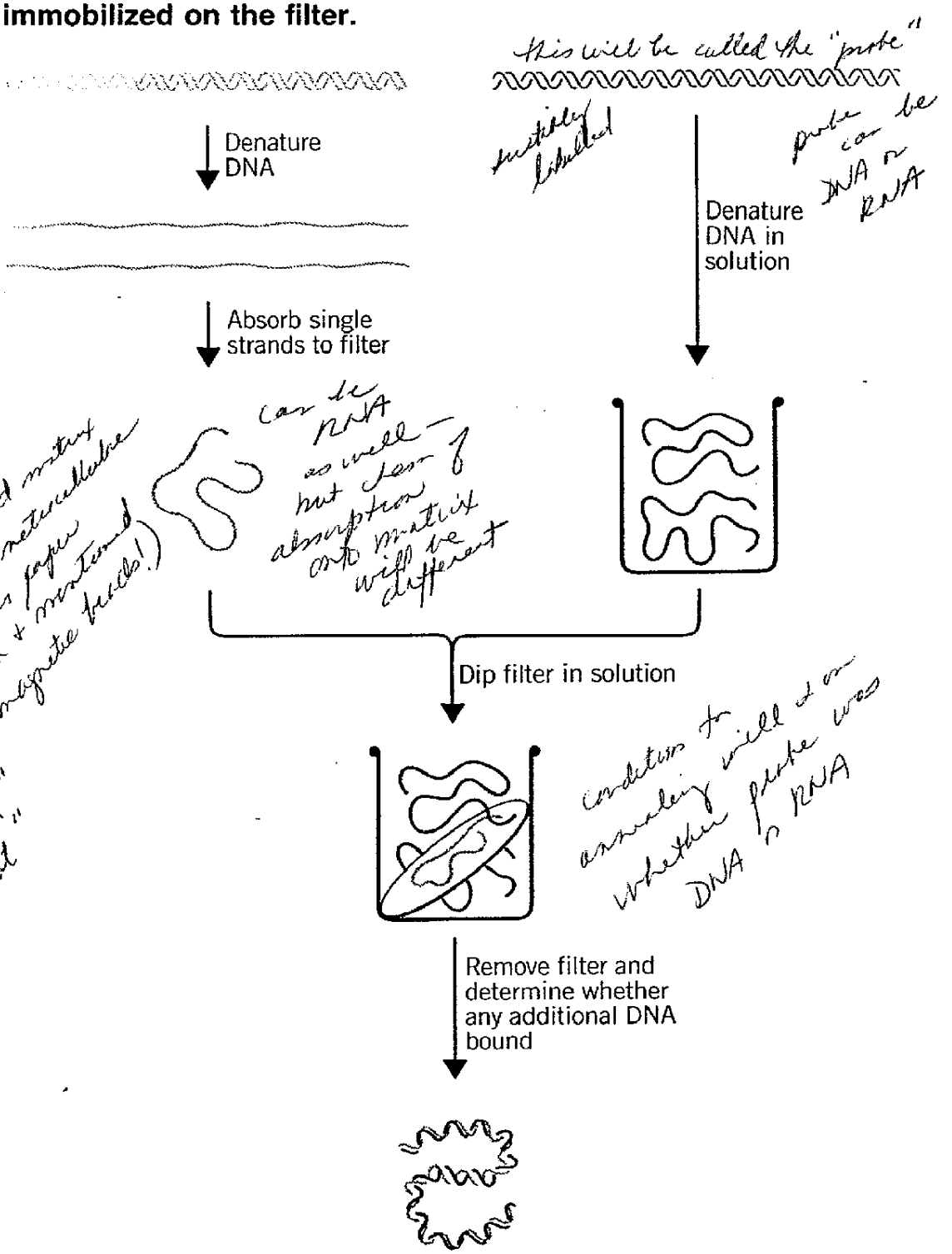
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Tests for base pairing between two single stranded polynucleotides

FIG 11

Filter hybridization establishes whether a solution of denatured DNA (or RNA) contains sequences complementary to the strands immobilized on the filter.



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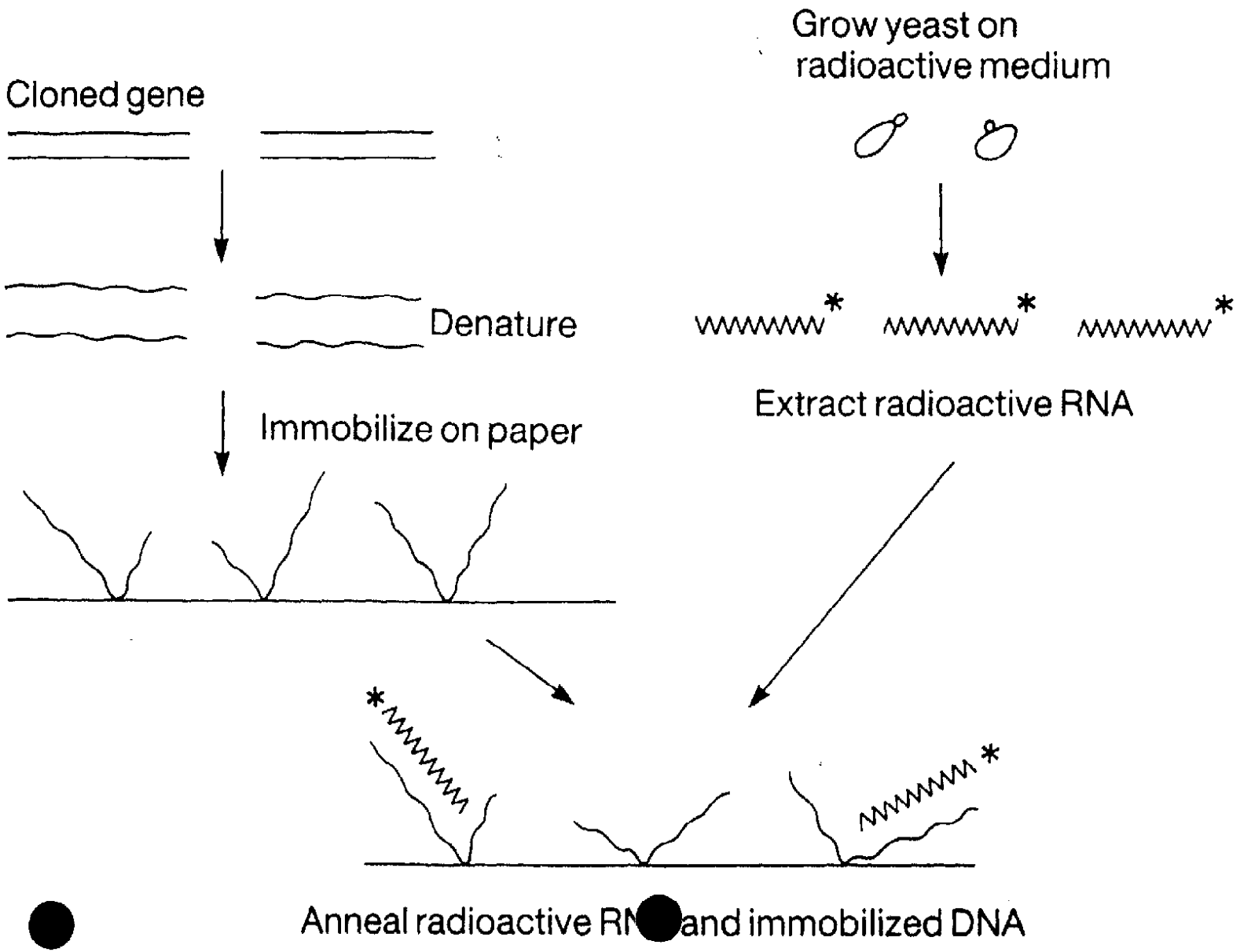
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 12 - A SCHEMATIC PRESENTATION OF A FILTER HYBRIDIZATION EXPERIMENT

Schematic of hybridization to quantitate a specific RNA.



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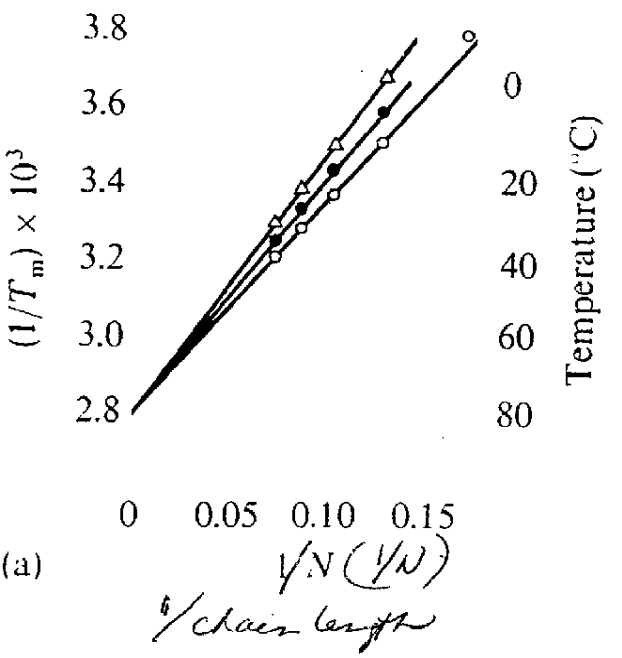
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 13 - FOR SHORT MOLECULES, DNA DUPLEX STABILITY IS A FUNCTION OF THEIR CHAIN LENGTH.



(a)

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 14A- DNA DUPLEXES ARE DESTABILIZED BY MISMATCHED BASE PAIRS.

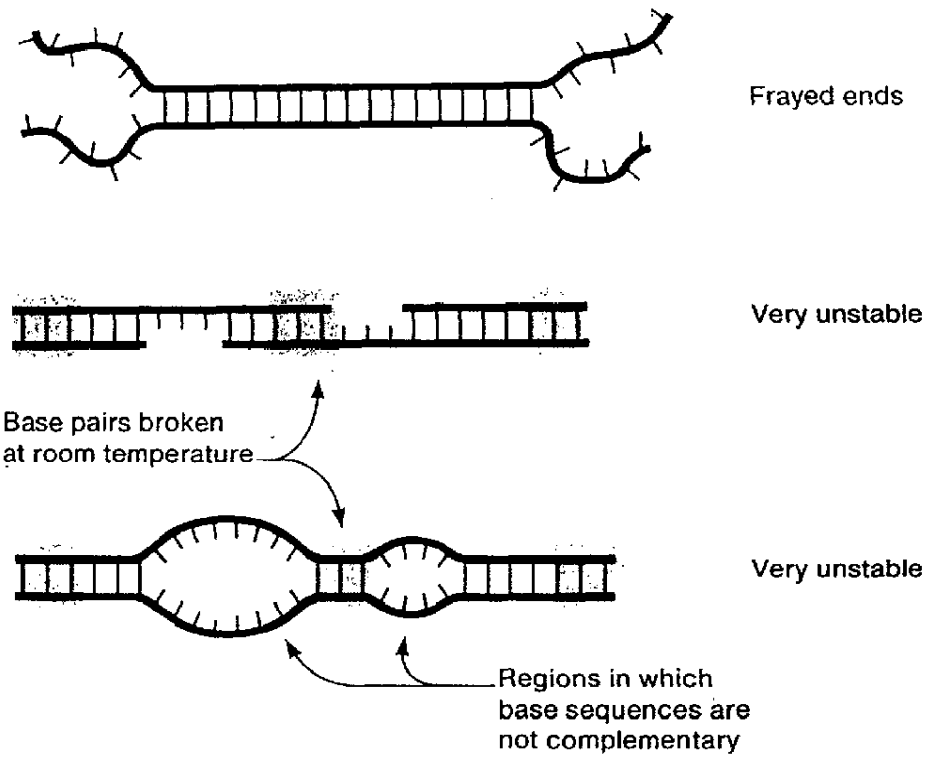

FIG 14B- DUPLEX STABILITY IS A FUNCTION IS A FUNCTION OF THE BASE COMPOSITION OF DNA (AND SOLUTION CONDITIONS).

↑GC ⇒ ↑ T_M due to extra H bond from GC

Also relevance to base stacking — π/π interactions

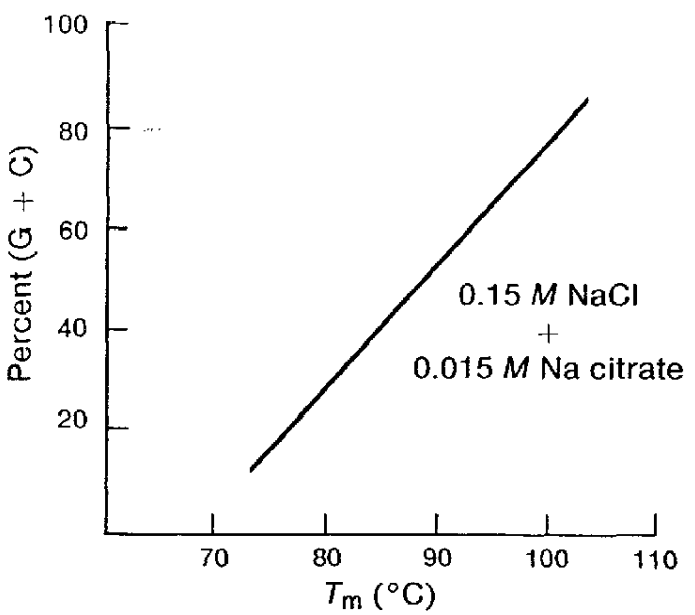

Figure 4-7
Plot of T_m versus G+C content of various DNA molecules.

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 15 - GENE EXPRESSION OCCURS IN TWO STEPS, TRANSCRIPTION (MRNA SYNTHESIS) AND TRANSLATION (PROTEIN SYNTHESIS).

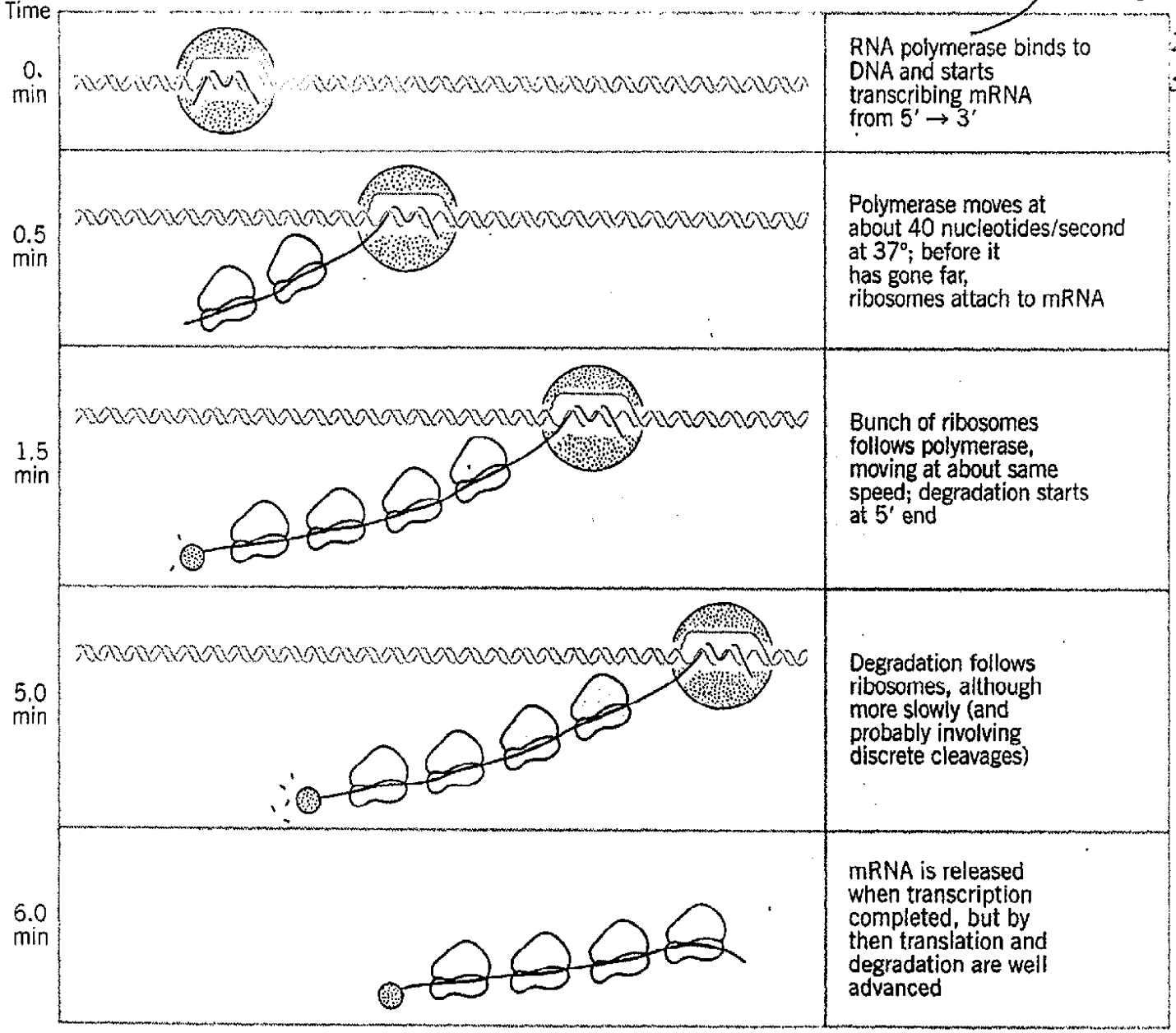

Figure 9.1

Transcription, translation, and degradation of mRNA all proceed simultaneously in bacteria.

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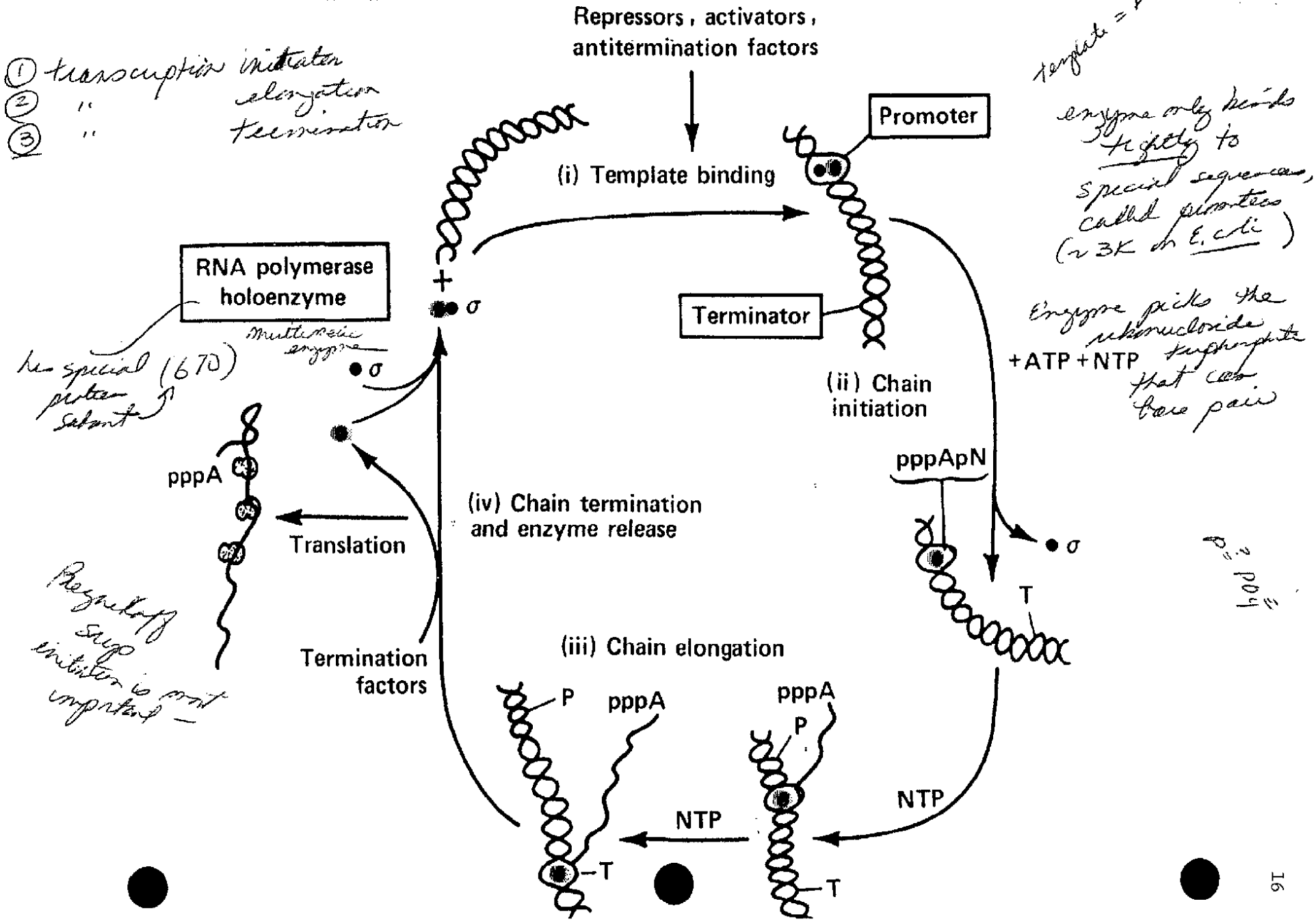
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

= complete enzyme

FIG 16 - THE TRANSCRIPTION CYCLE INCLUDES INITIATION OF TRANSCRIPTION BY HOLOENZYME AT PROMOTERS, ELONGATION OF THE MESSAGE BY CORE ENZYME, AND TERMINATION.



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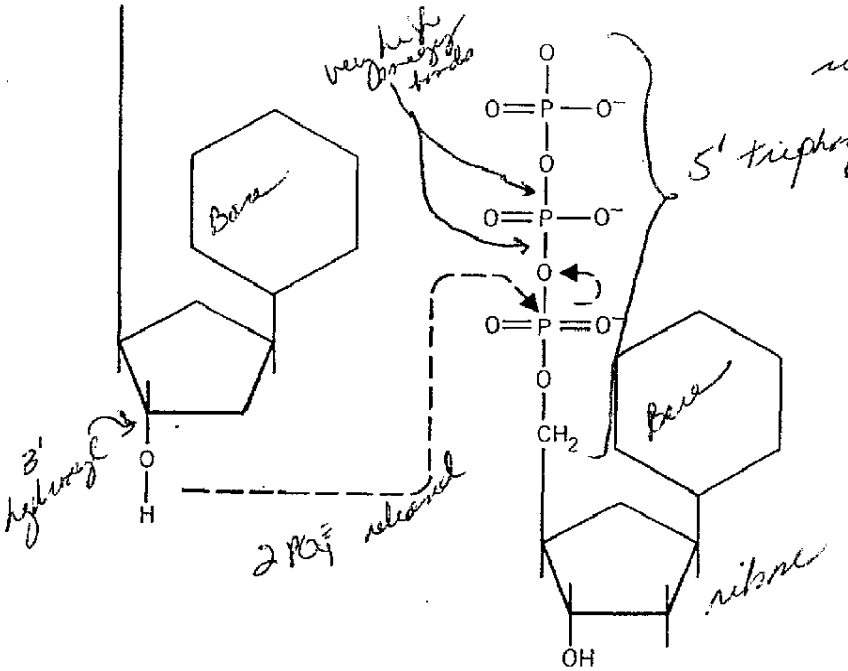
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 17A- MECHANISM OF RNA PHOSPHODIESTER BOND FORMATION DURING RNA SYNTHESIS.



ribonucleoside triPi are substrate for RNA synthesis

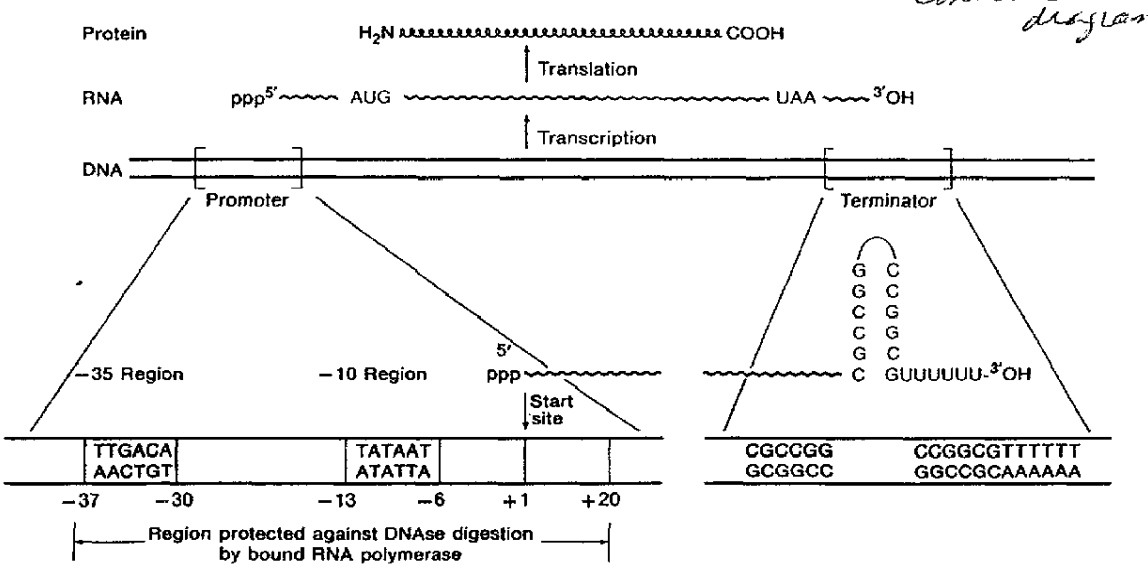
phosphate as "petrochemical" of the cell — except its reservoir

condensation doesn't know which bases are being picked up

Figure 10.4
Phosphodiester bond formation involves a hydrophilic attack by the 3′-OH group of the last nucleotide of the chain on the 5′ triphosphate of the incoming nucleotide, with release of pyrophosphate.

FIG 17B- A DNA SEQUENCE SIGNAL (THE PROMOTER) STIMULATES TRANSCRIPTION INITIATION; AN RNA SEQUENCE SIGNAL STIMULATES TERMINATION.

RNA couple to bottom strand, identical to top (except T→U) as shown by convention in most diagrams



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 18A- COMPARATIVE SEQUENCE ANALYSIS INDICATES THE FAVORED PROMOTER SEQUENCE FOR E. COLI RNA POLYMERASE. OTHER RNA POLYMERASES RECOGNIZE OTHER SEQUENCES.

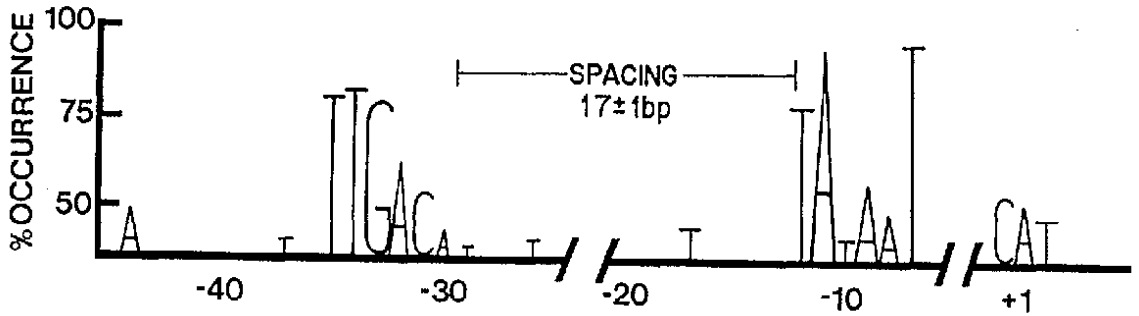
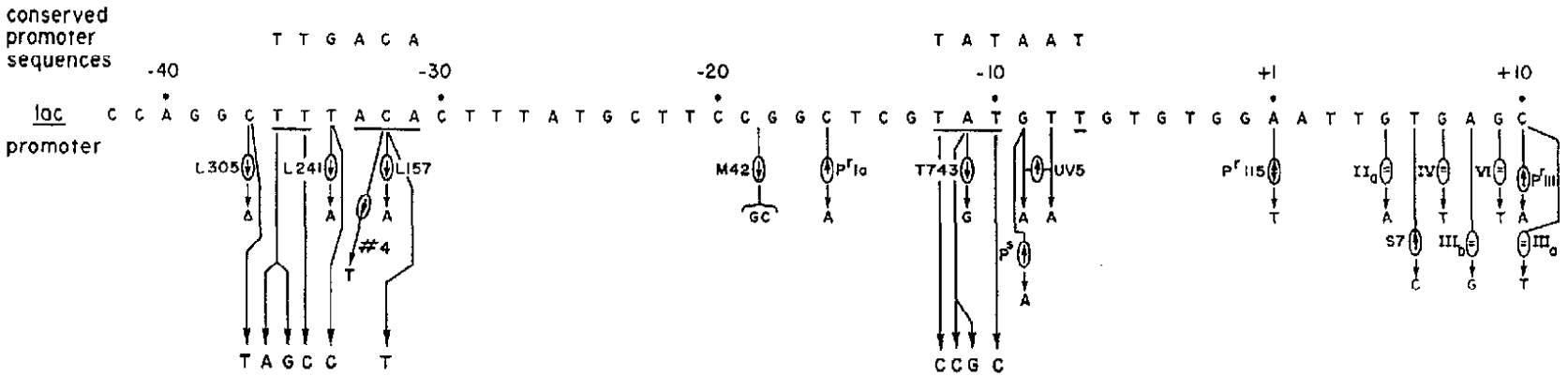


FIG 18B- GENETIC ANALYSES CONFIRM THE IMPORTANCE OF PROMOTER SEQUENCE.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 19A- GENE EXPRESSION IS OFTEN REGULATED. LAC OPERON EXPRESSION IS INDUCED BY ADDING -GALACTOSIDE COMPOUNDS TO THE CULTURE.

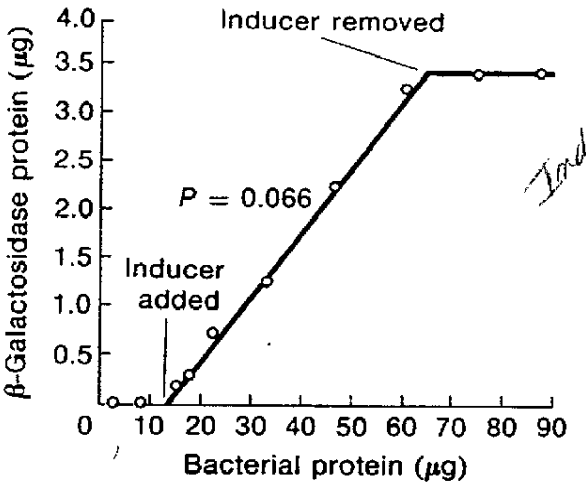

FIG 19B- SCHEMATIC OF THE LAC OPERON. THE REPRESSOR REGULATES BY BINDING TO THE OPERATOR AND BLOCKING TRANSCRIPTION INITIATION. THE REPRESSOR IS INACTIVATED BY INDUCER BINDING.

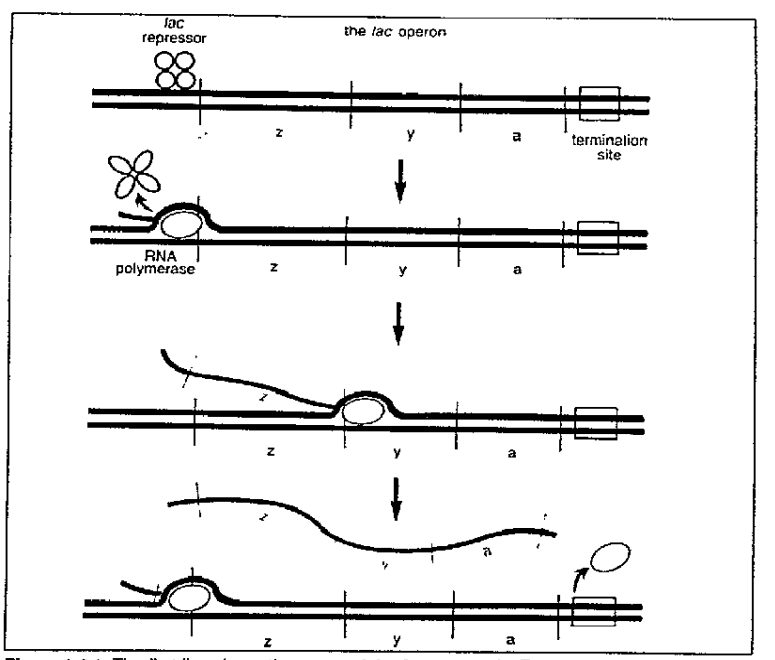

Figure 1.4.1 The first line shows the genes of the *lac* operon. *lacZ*, *lacY*, and *lacA* transcription is repressed by *lac* repressor. In the next line, inactive repressor is no longer bound to the operator, and the genes are being transcribed by RNA polymerase. In the third line, RNA polymerase transcribes the operon. Finally, RNA polymerase encounters a transcription terminator and, in a reaction requiring rho protein, RNA polymerase is detached from the DNA and the nascent transcript.

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

∃ a specific operator (DNA sequence) for each repressor (check)

FIG 20- THE DNA SEQUENCE OF THE LAC CONTROLLING ELEMENTS SHOWS THAT THE REPRESSOR BINDING SITE OVERLAPS WITH THE RNA POLYMERASE BINDING SITE. THE REPRESSOR BINDING SITE (THE OPERATOR) IS DEFINED BY MUTATIONS.

repressor bending to operator sterically interferes w/ RNA polymerase bending to promoter

lac CONTROLLING ELEMENTS

← CAP SITE → ← PROMOTER → ← OPERATOR →

-35 -10 +1

TGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATG

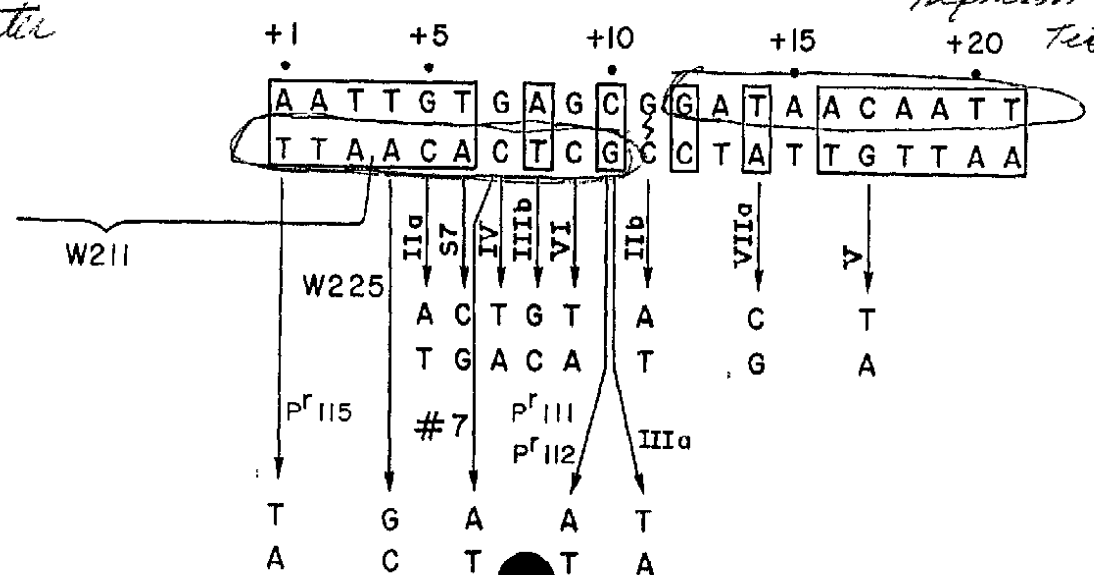
ACTCGCGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTAACACTCGCCTATTGTTAAAGTGTGTCCTTTGTCGATAC

UGA(lacI mRNA) pppAAUUGU-----(lac mRNA)------AGGA-------AUG

lac Operator

ing like promoter

note mirror image of circled areas
Repressor is ~~mirror image~~ Tetramers that are mirror images — ∴ it all fits



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Translation: polynucleotide to polypeptide – not simple base pairing info

"decoding"

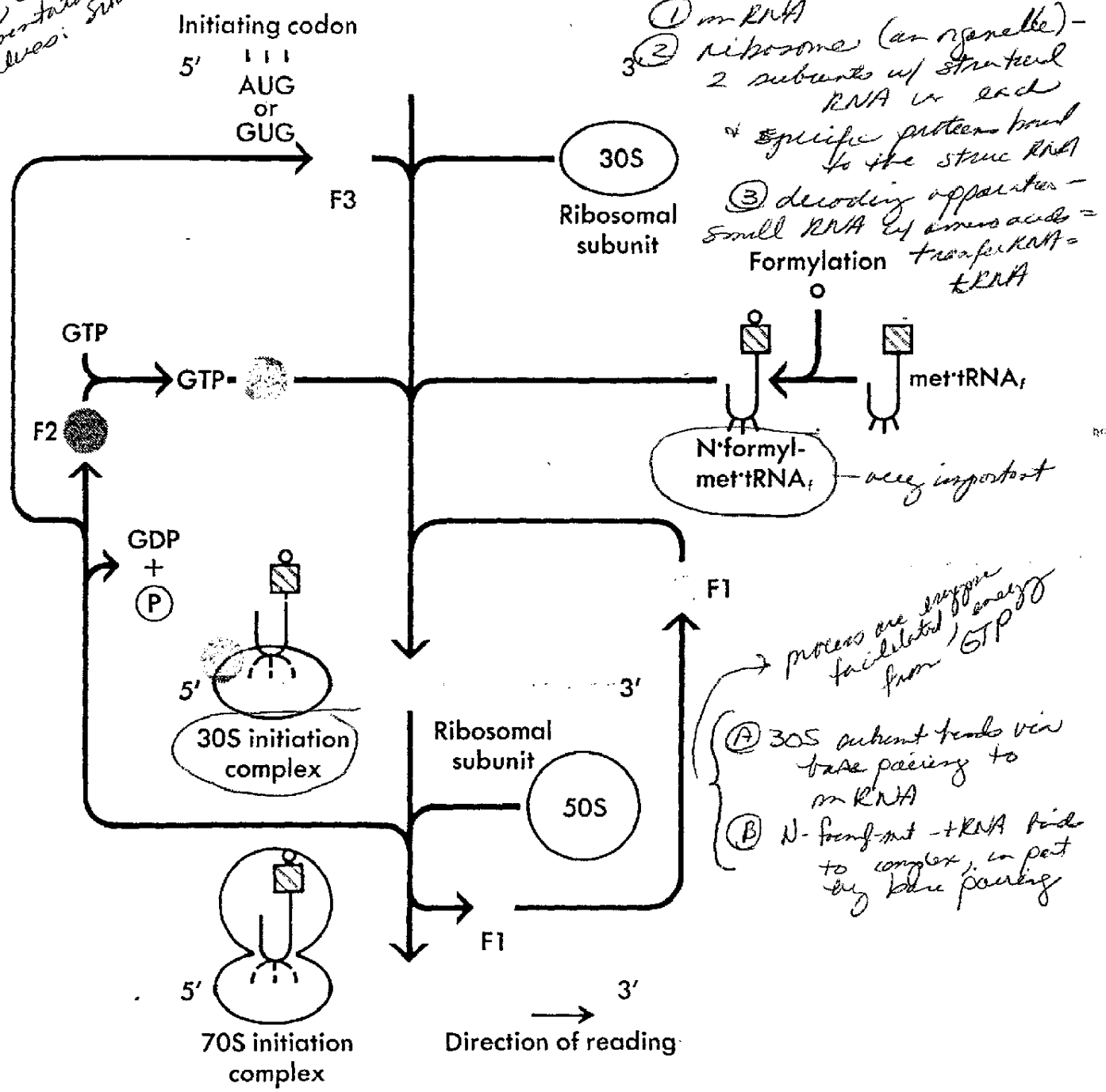
Biochemistry more complex than transcription

FIG 21- DIAGRAMMATIC VIEW OF THE TRANSLATION INITIATION PROCESS.

30S, 50S = sedimentation rate values; Svedberg #s

3 major participants in translation initiation

1. mRNA
2. ribosome (an organelle) – 2 subunits w/ structural RNA in each & specific proteins bound to the struc RNA
3. decoding apparatus – small RNA by amino acids = transfer RNA = tRNA



→ process are energy facilitated / energy from GTP

(A) 30S subunit binds via base pairing to mRNA

(B) N-formyl-met-tRNA binds to complex, in part by base pairing

Ribosome compared to factory – where it happens

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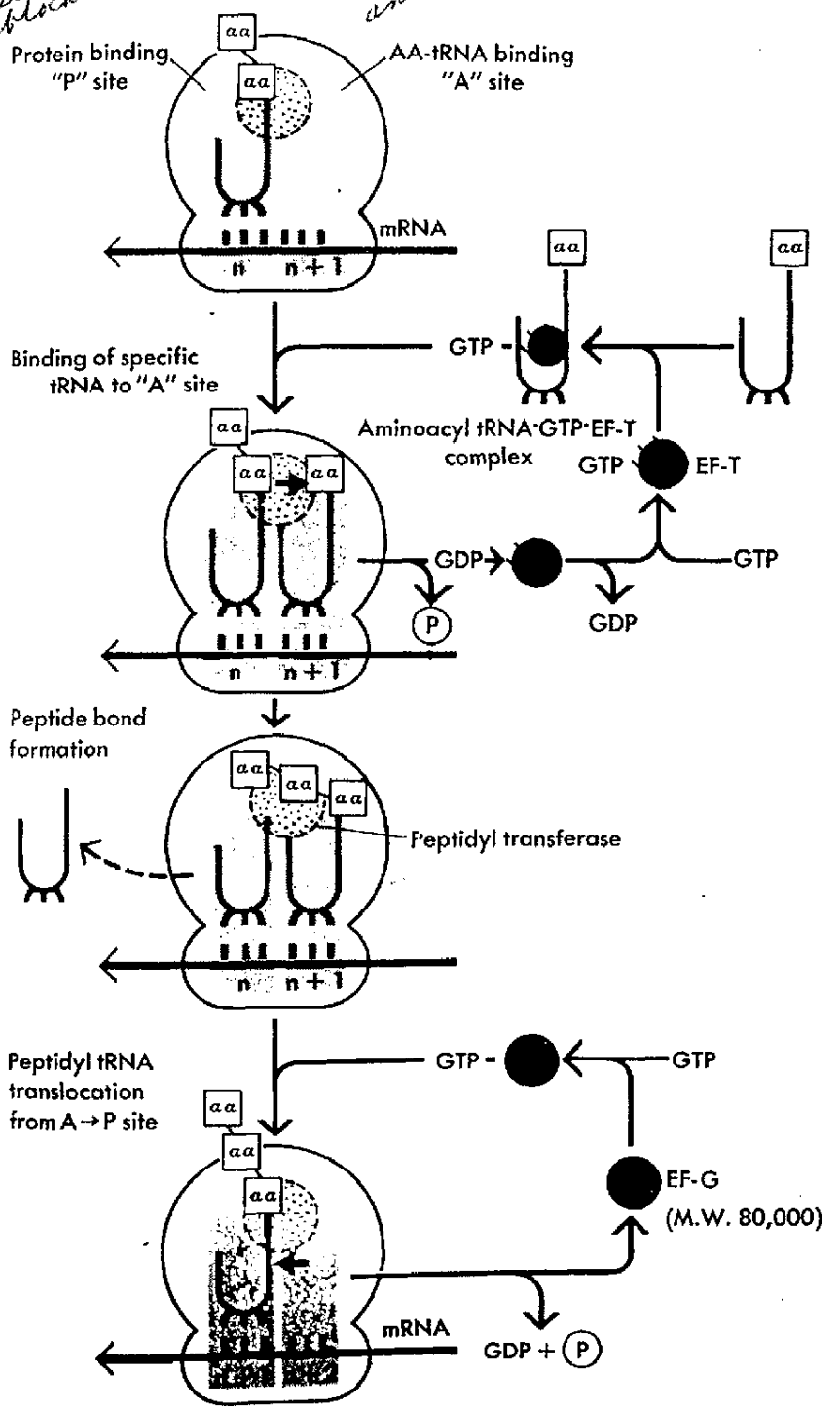
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

carboxy terminus linked to tRNA in formylmet tRNA
Synthesis is N terminus to carboxy terminus

FIG 22 - THE TRANSLATION ELONGATION PROCESS.

tRNAs bind here if N-terminus is blocked

amino acid = AA?



Growing polypeptide chain, attached by the terminal tRNA group to the protein binding site.

Attachment of specific aminoacyl tRNA molecule by hydrogen bonding to n + 1 codon of mRNA chain. This requires elongation factor T and ATP.

Formation of peptide bond between AA_2 and AA_3 by peptidyl transferase, an integral protein of the 50S subunit.

Ejection of tRNA from 'P' site

Movement of growing polypeptide chain from "A" site to protein binding site. This requires elongation factor G and GTP.

Simultaneous movement of mRNA to place (n + 2) codon at the "A" site.

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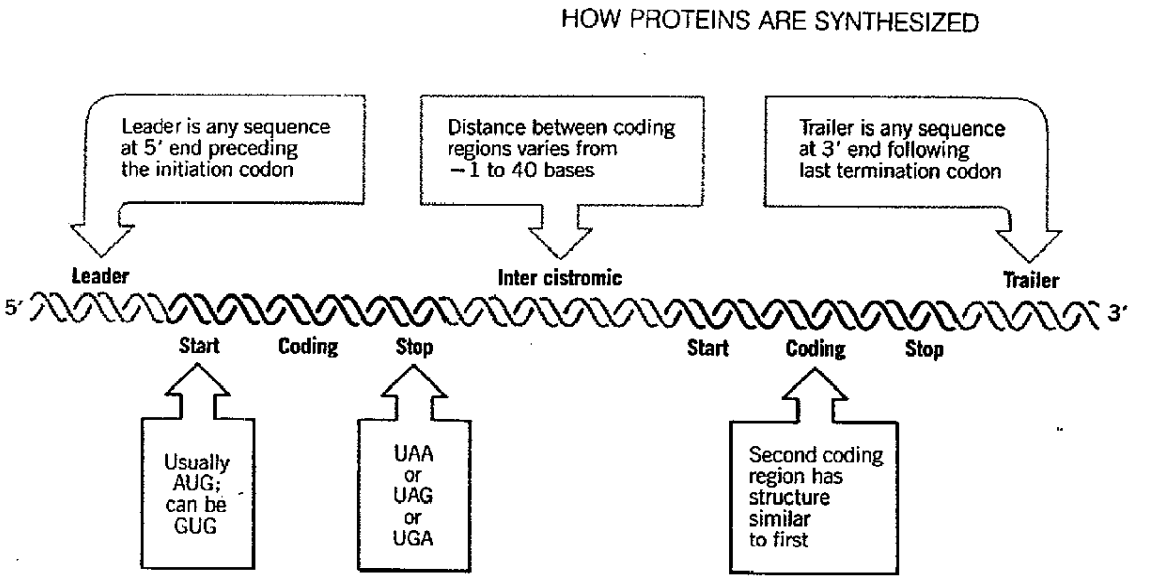
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 23a - A PROTEIN CODING REGION IS DEFINED BY A START SIGNAL (INCLUDING AN AUG) AND A STOP SIGNAL (UAG OR UAA OR UGA).



MUTATIONS WHICH AFFECT THE SHINE & DALGARNO SEQUENCE

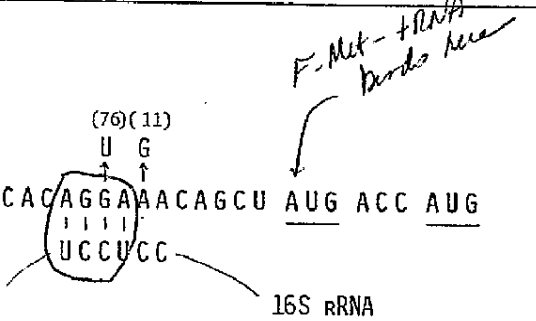


FIG 23b - THE TRANSLATION START SIGNAL ALSO INCLUDES A "SHINE-DALGARNO" SEQUENCE (PARTIALLY HOMOLOGOUS TO 16S RIBOSOMAL RNA).

MUTATION	UNITS B-GAL	% λPLAC:361
WT	657±35	100
76	45±1	7.5
11	1810±25	275

CONCLUSIONS:

STRENGTH OF THE INTERACTION BETWEEN THE mRNA AND THE rRNA STRONGLY EFFECTS TRANSLATION INITIATION

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

code is universal triplet redundant

FIG 24a - mRNA TRANSLATES PROTEINS THROUGH A TRIPLET CODE.

circled - no degeneracy

Degree of redundancy does relate to use of amino acid - at least start.

Table 13–7 The Genetic Code

First Position (5′ End)	Second Position U	C	A	G	Third Position (3′ End)
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Term[a]	Term	A
	Leu	Ser	Term	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	GluN	Arg	A
	Leu	Pro	GluN	Arg	G
A	Ileu	Thr	AspN	Ser	U
	Ileu	Thr	AspN	Ser	C
	Ileu	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

[a] Chain terminating (formerly called "nonsense").

UAA, UAG, UGA = termination codons

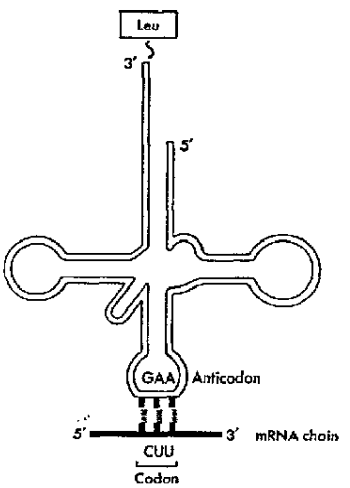
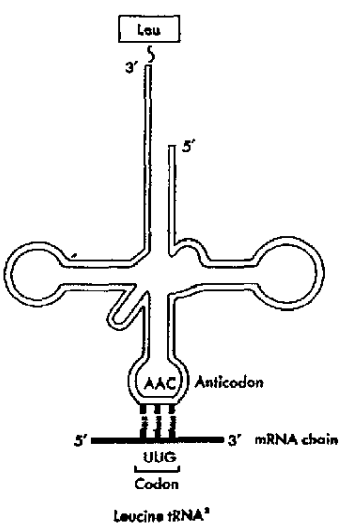


FIG 24b - tRNA IS THE DECODING OR ADAPTER MOLECULE.

~150 b.p.
Complex 2° structure
lot of X-ray data avail



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 25- CODE WORDS ARE READ SEQUENTIALLY; AN ADDITION OR DELETION OF 1 OR 2 BASES COMPLETELY CHANGES THE SUBSEQUENT READING OF THE MESSAGE BY CHANGING THE READING FRAME.

frameshift mutations

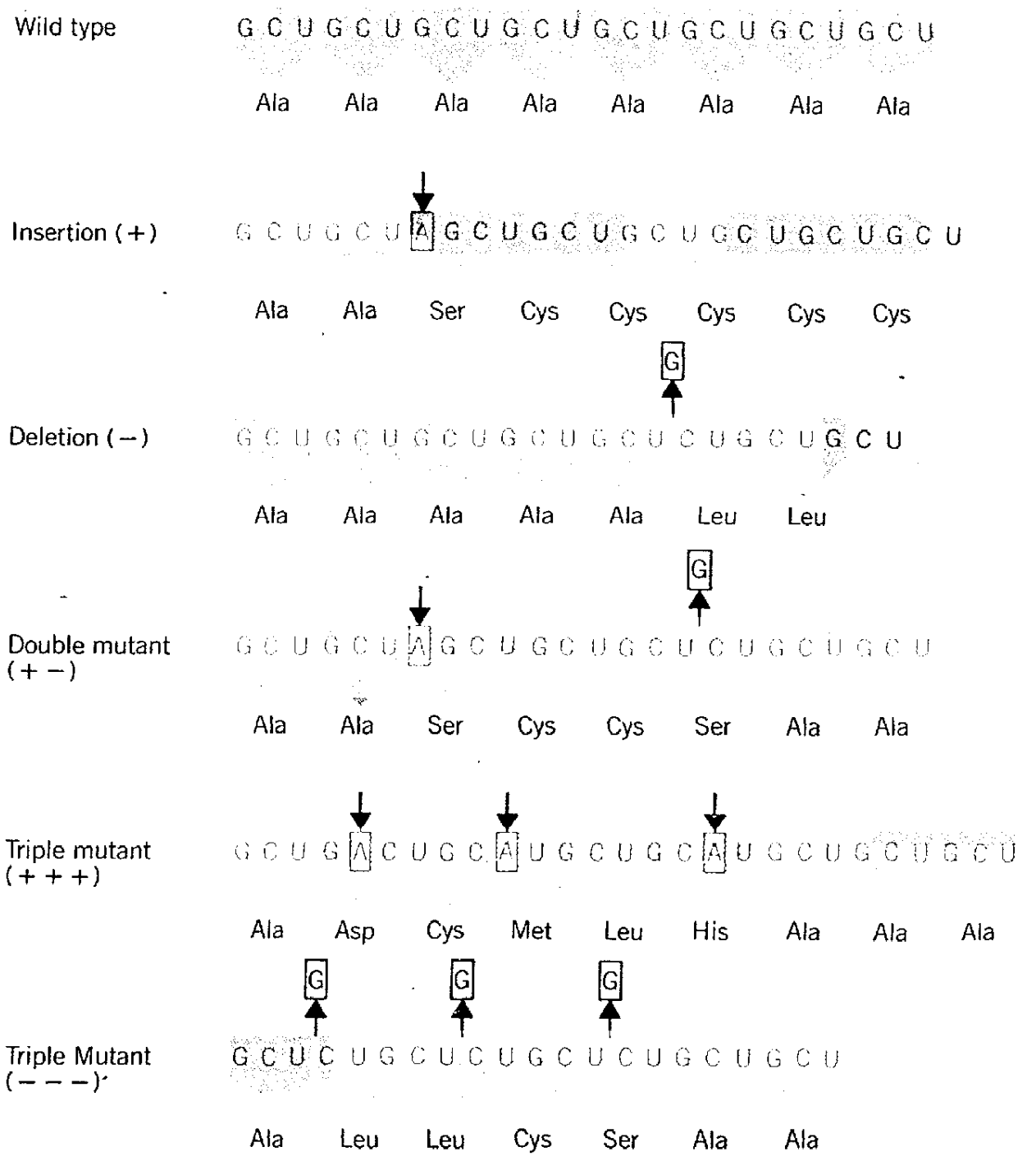

Figure 4.3
Frameshift mutations show that the genetic code is read in triplets from a fixed starting point.

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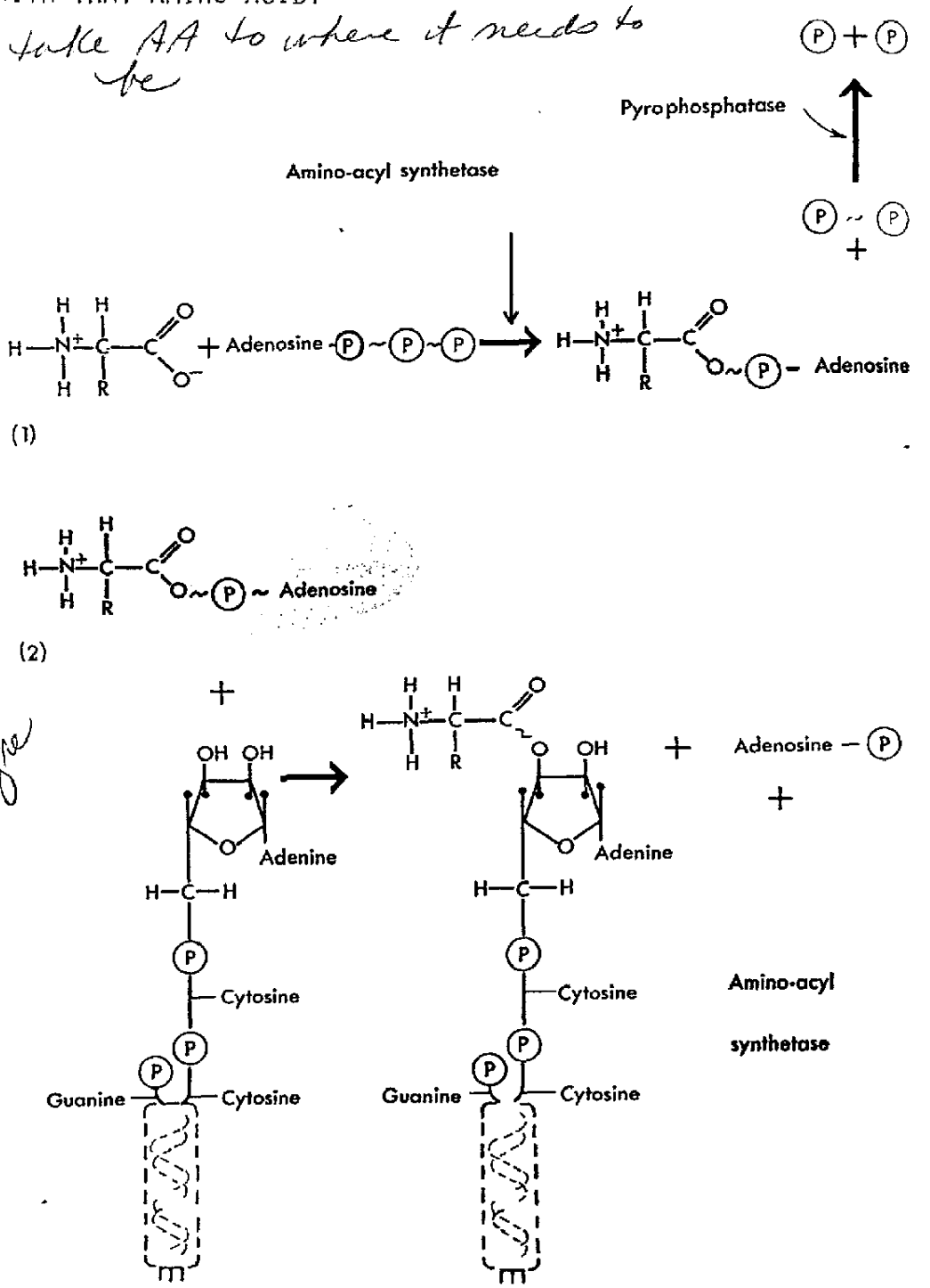
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Taking the amino acids & organizes them as polypeptide! AAs from dietary organ as I recall

FIG 26- FOR EACH tRNA THERE IS A SPECIFIC AMINO-ACYL SYNTHETASE ENZYME WHICH ACTIVATES THE APPROPRIATE AMINO ACID AND THEN CHARGES THE tRNA WITH THAT AMINO ACID.

tRNA take AA to where it needs to be



no proofreading of AA once they're "charged"

adenylic acid residue of the tRNA.

$$AA \sim AMP + tRNA \underset{}{\overset{\text{amino acyl synthetase}}{\rightleftharpoons}} AA \sim tRNA + AMP \qquad (12\text{-}2)$$

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

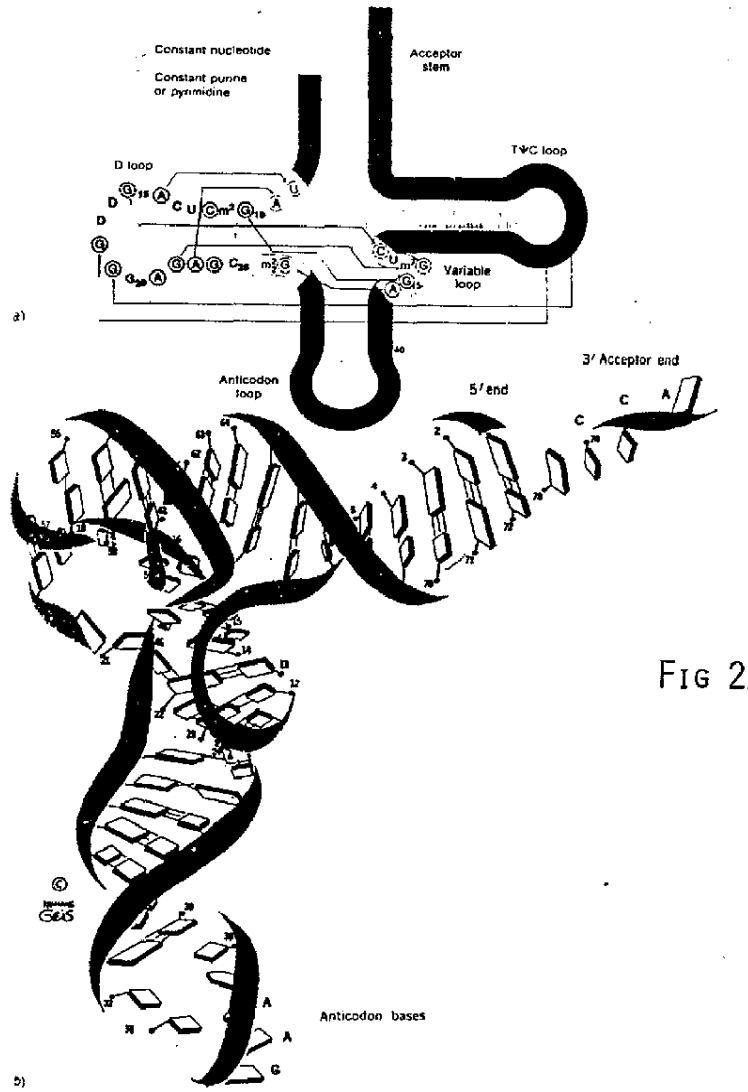


FIG 27A- TRNA STRUCTURE.

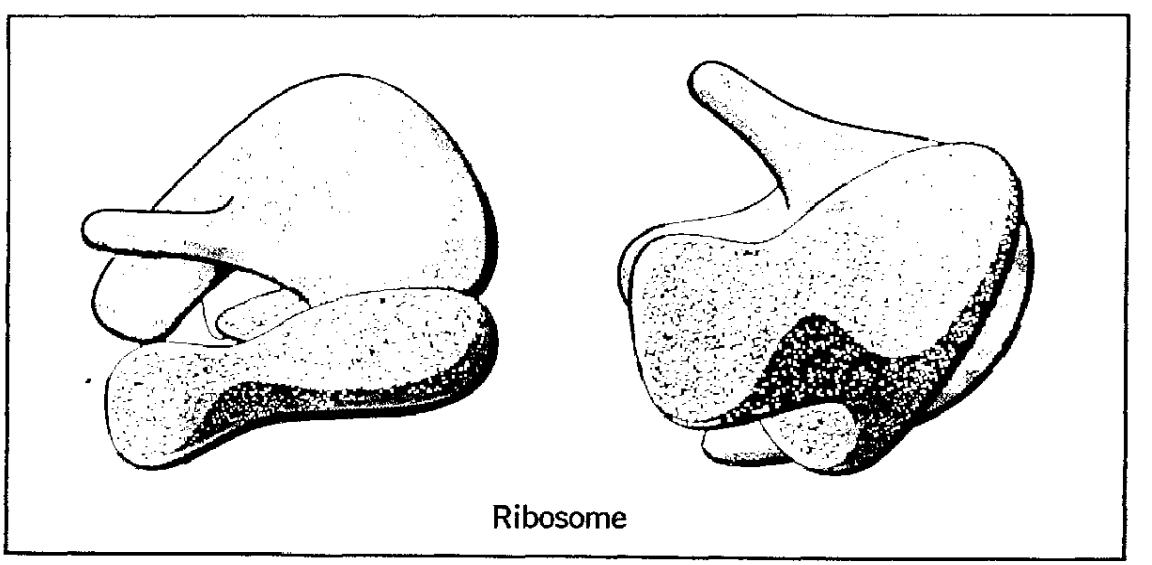
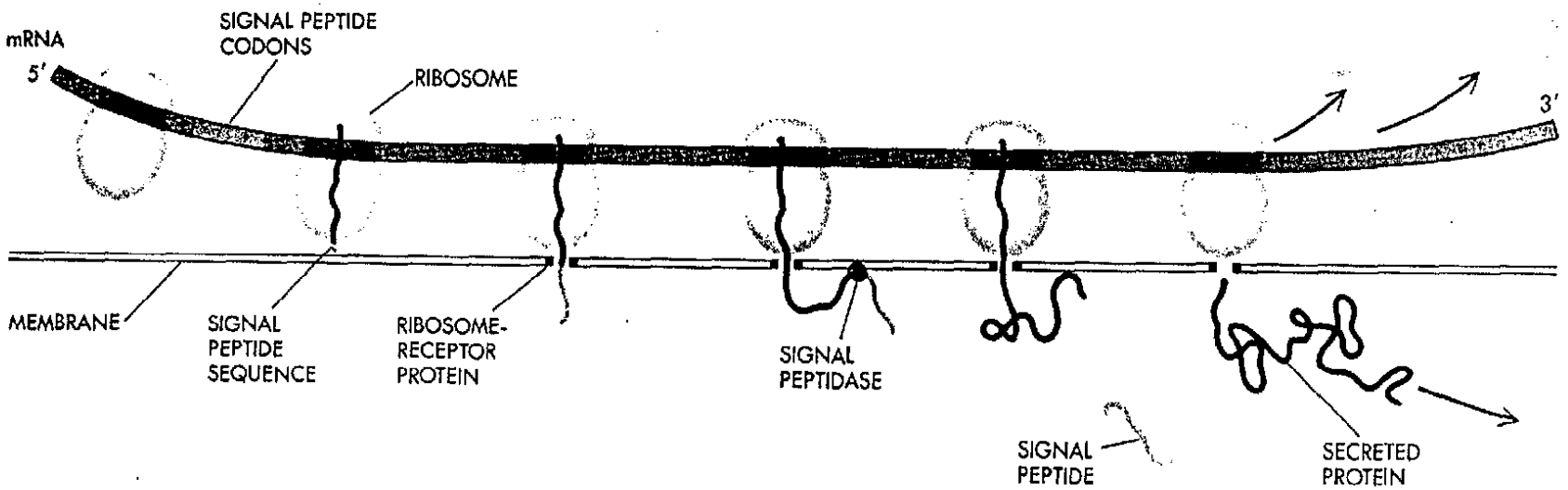


FIG 27B- RIBOSOMES (COMPOSED OF PROTEINS AND RNA) PLUS ENZYMES ARE THE TRANSLATION MACHINERY.

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 28 - SOME PROTEINS ARE ALSO PROCESSED BY REMOVAL OF SIGNAL SEQUENCES.



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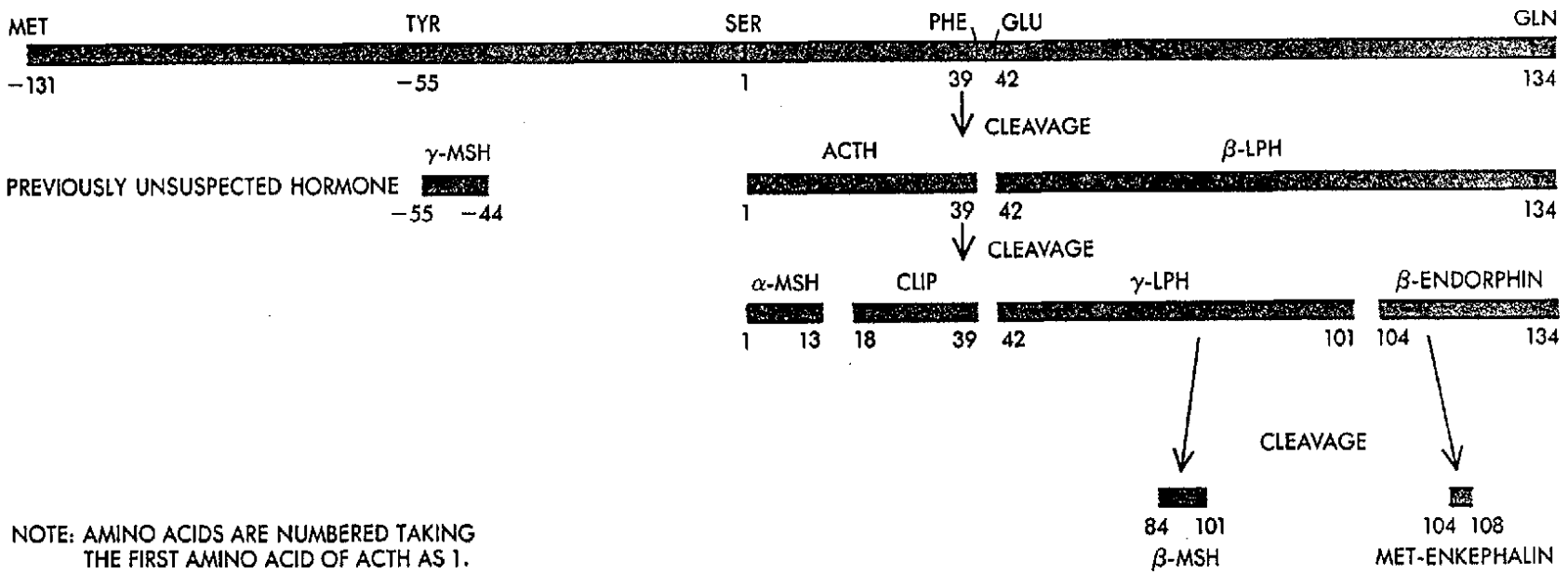
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Make one big protein + cut up later

FIG 29 - IN EUKARYOTES SOME NEWLY MADE PROTEINS ARE CUT TO YIELD MORE THAN ONE FINISHED PROTEIN.

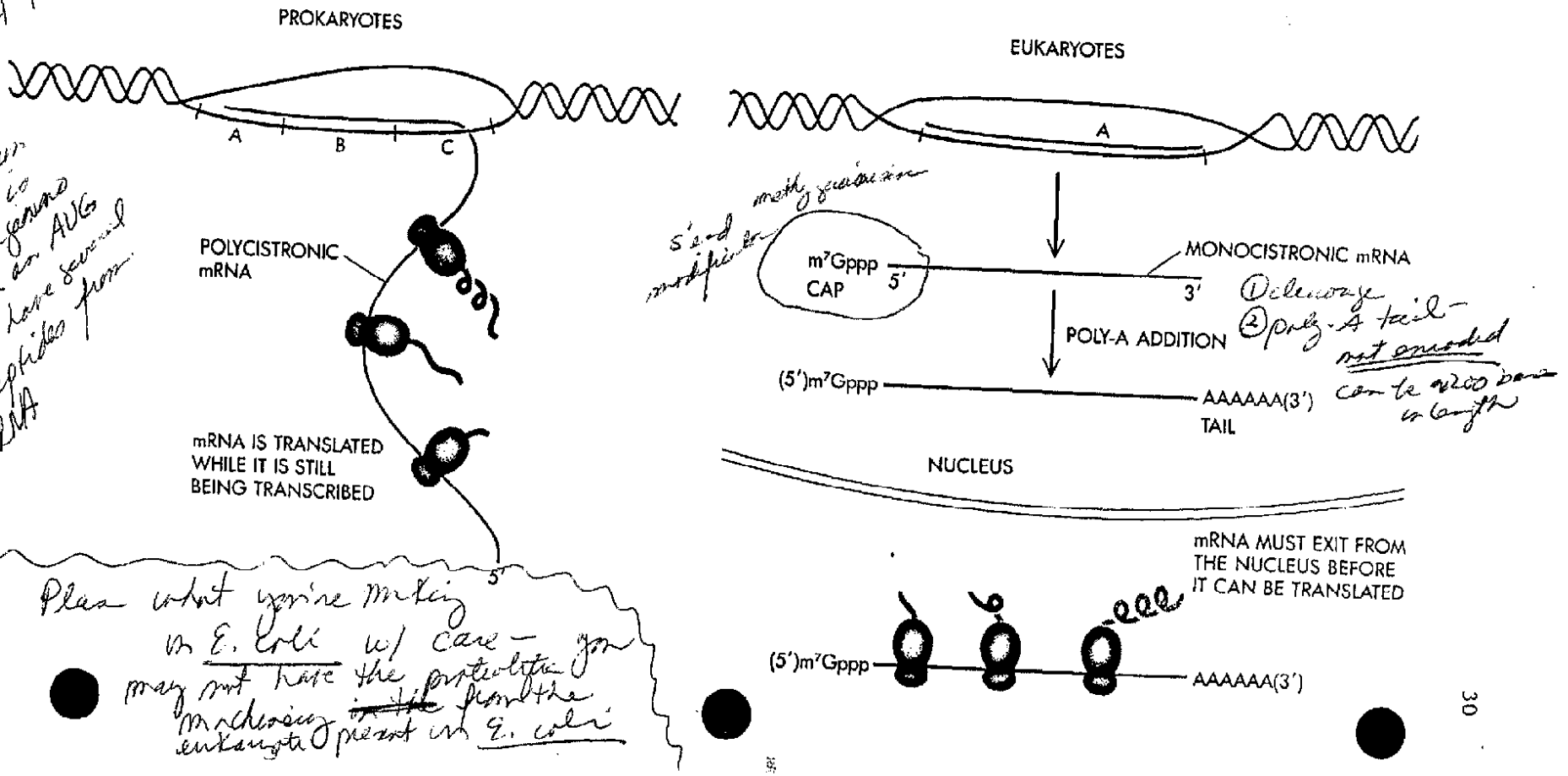


NOTE: AMINO ACIDS ARE NUMBERED TAKING THE FIRST AMINO ACID OF ACTH AS 1.

PM30011931O6

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 30- IN PROKARYOTES TRANSCRIPTION AND TRANSLATION ARE COUPLED; IN EUKARYOTES THEY OCCUR IN DIFFERENT CELL COMPARTMENTS AND THE mRNA UNDERGOES EXTENSIVE MODIFICATION.



PM30011931O7

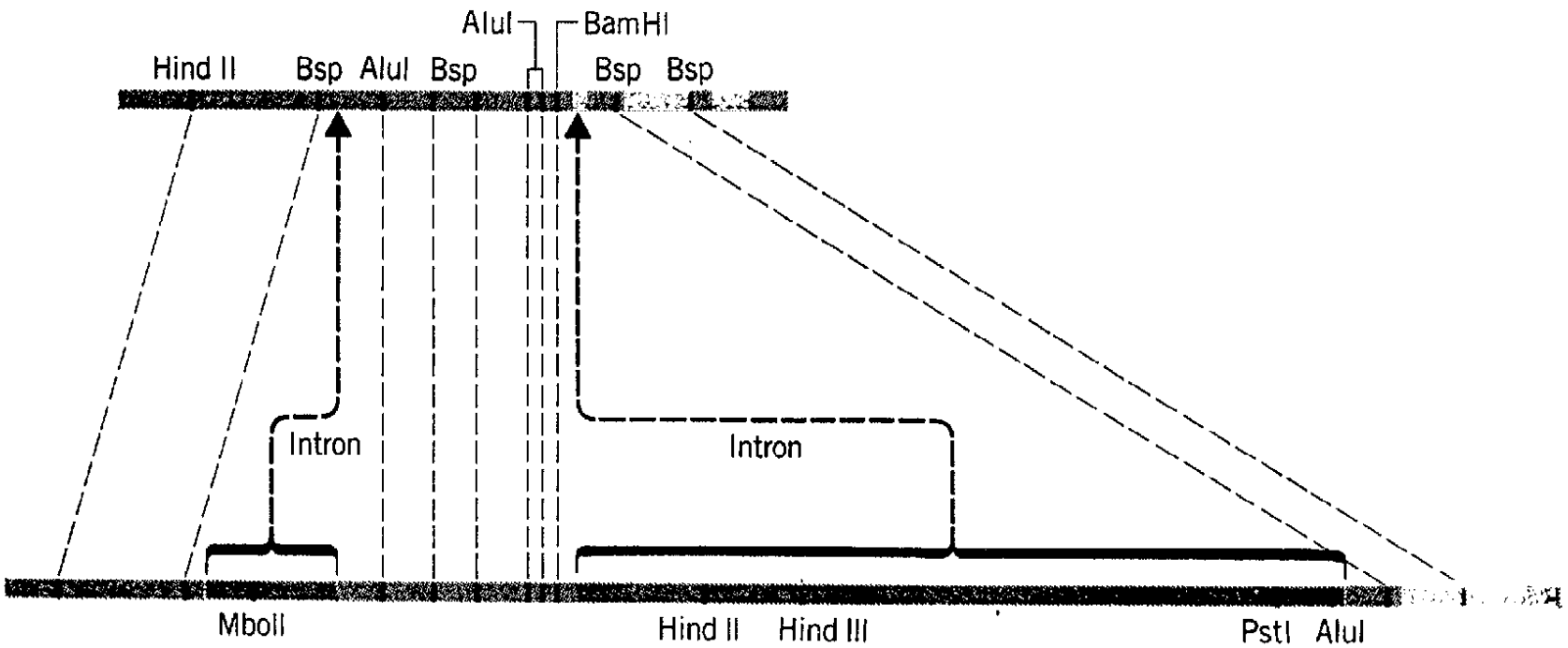
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Yeast E coli doesn't have enzymes needed to remove the introns

Nuclear (chromosomal) DNA includes DNA which encodes messages + proteins = exons
also " sequences which do neither = introns
Introns can be 90%

FIG 31 - IN EUKARYOTES (BUT NOT IN PROKARYOTES) MOST GENES CONTAIN INTERVENING SEQUENCES NOT CODING FOR PROTEIN. SUCH SEQUENCES ARE REMOVED FROM THE mRNA BY SPLICING STEPS.

CONSTITUTION OF THE EUCARYOTIC GENOME



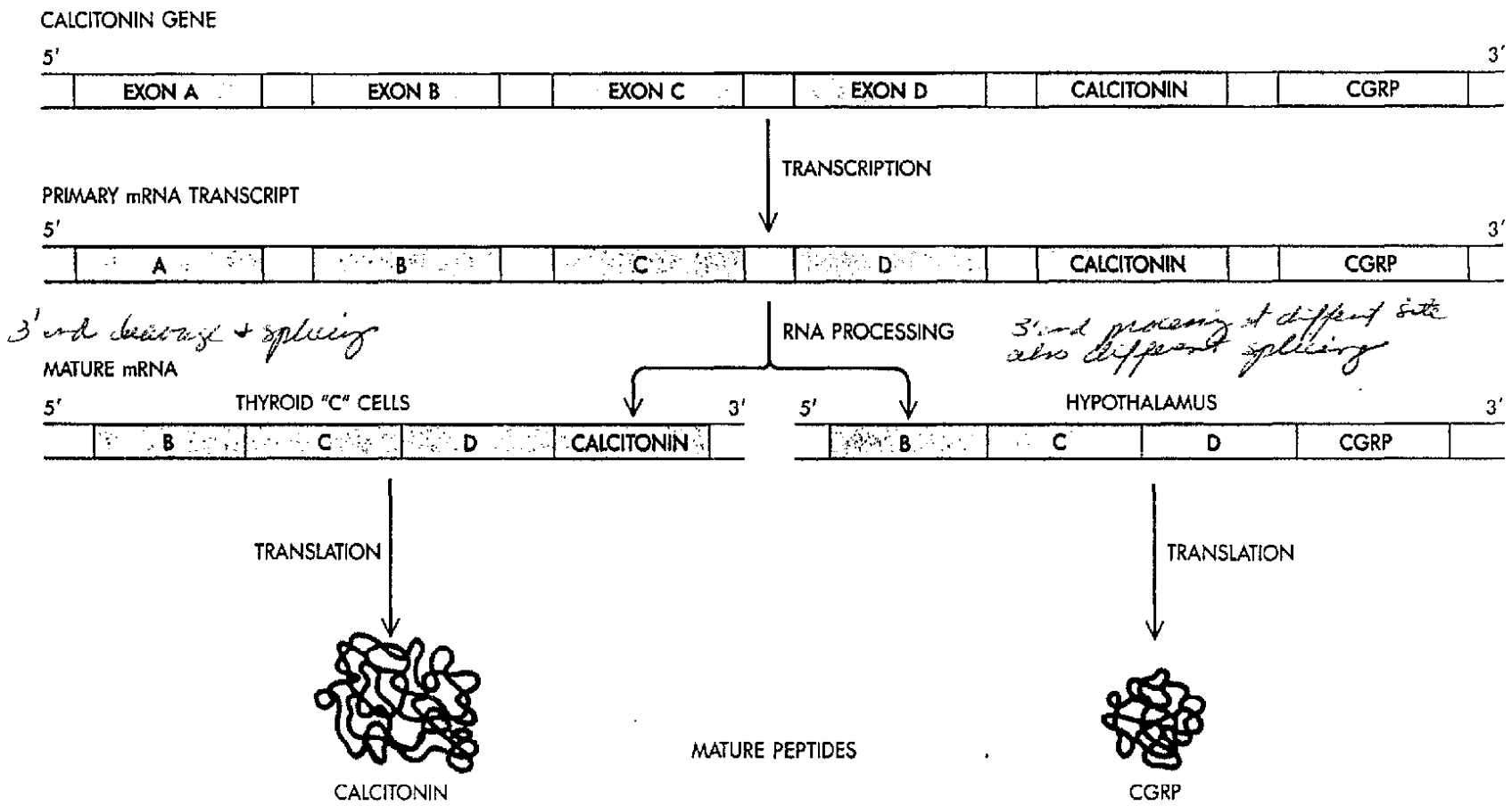
How to get rid of introns before doing work in bacteria

PM3001193108

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

other examples are immunoglobulins
- introns allows proteins which are closely related to be spliced by a sequence

FIG 32- ALTERNATIVE SPLICING OCCURS FOR SOME EUKARYOTIC mRNA'S YIELDING DIFFERENT MATURE mRNA'S (AND PROTEINS).



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

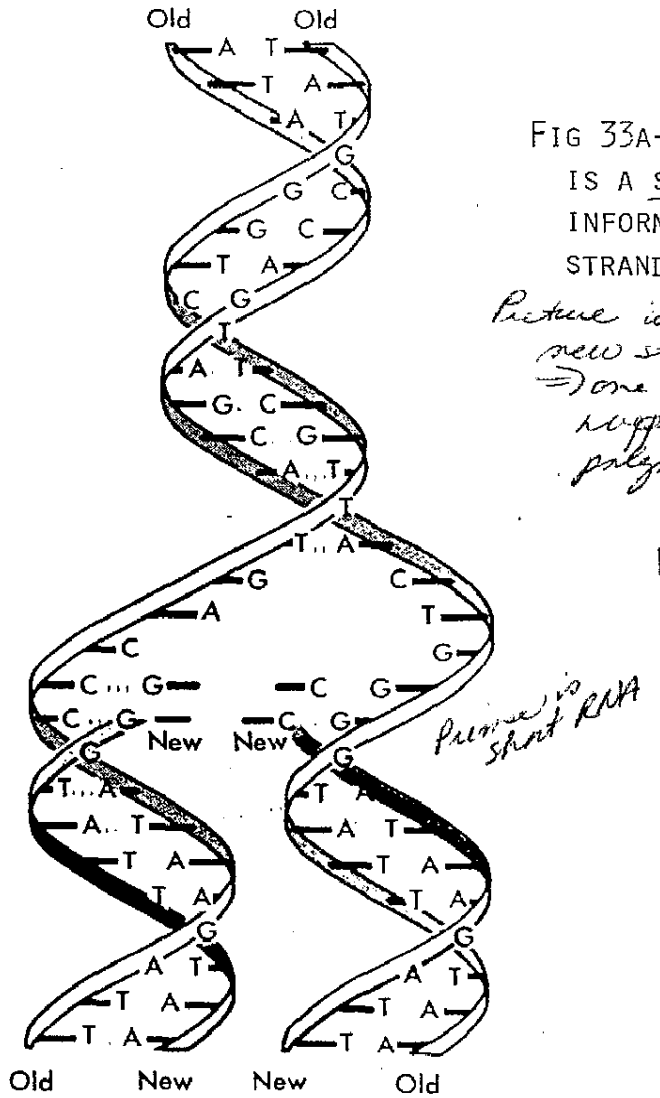


FIG 33A-GENE DUPLICATION (DNA SYNTHESIS) IS A SEMI-CONSERVATIVE PROCESS INVOLVING INFORMATION TRANSFER BY BASE PAIRING AND STRAND SEPARATION.

Picture is wrong in that it shows both new strands being made concurrently ⇒ one is 3'→5' and that does not happen. Always 5'→3' for polynucleotide polymerases

FIG 33B- DNA SYNTHESIS IS CATALYZED BY DNA POLYMERASE; ALL DNA POLYMERASES HAVE THREE PROPERTIES:

THEY CAN ONLY ELONGATE A PRE-EXISTING STRAND (PRIMER), ADDING NUCLEOTIDES TO A 3' OH END.

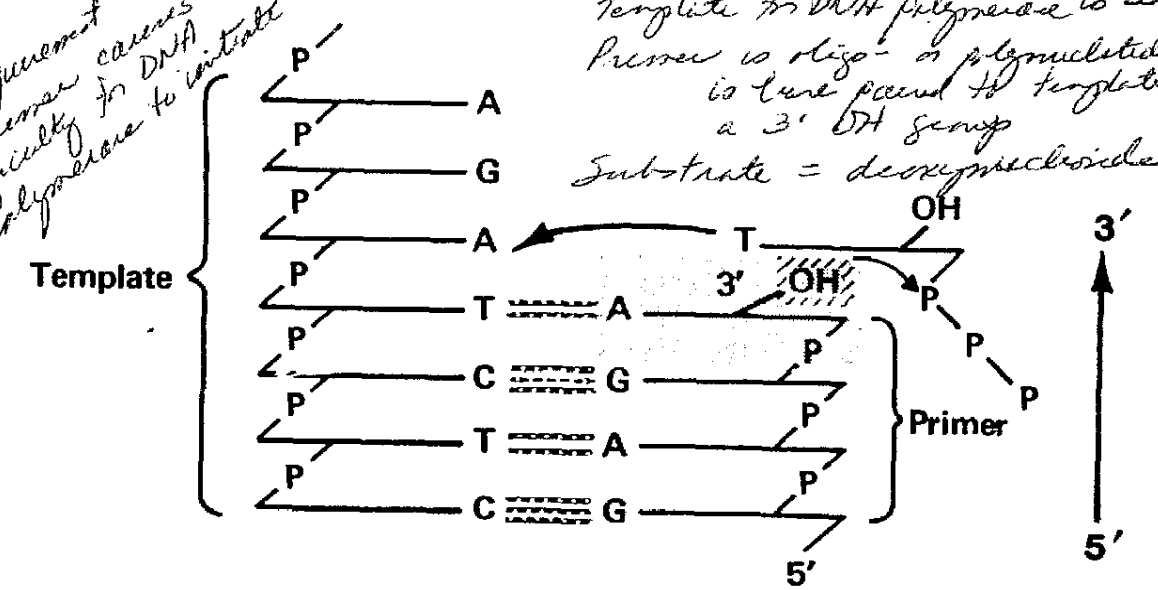
THEY FUNCTION IN ONLY ONE DIRECTION WITH A 5' TO 3' POLARITY.

THEY REQUIRE A TEMPLATE WHICH, THROUGH CMPLEMENTARY H BONDING RULES, DICTATES WHICH NUCLEOTIDE IS TO BE INCORPORATED.

Primer is short RNA

Requirement for primer causes difficulty for DNA polymerase to initiate

Template for DNA polymerase is ss DNA
Primer is oligo- or polynucleotide which is base paired to template + has a 3' OH group
Substrate = deoxynucleoside triPO4 =



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 34- THE UNIQUE POLARITY OF DNA POLYMERASES RESULTS IN THE SYNTHESIS OF A LEADING CONTINUOUS STRAND AND A LAGGING DISCONTINUOUS STRAND.

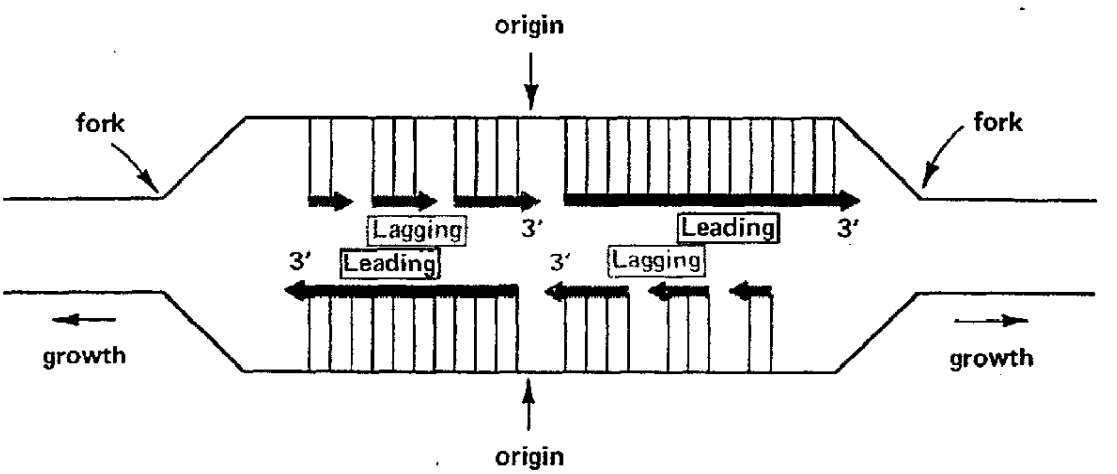

FIGURE 11-2
Bidirectional fork movement from the origin. Replication is semidiscontinuous: continuous on the leading strand and discontinuous on the lagging strand.

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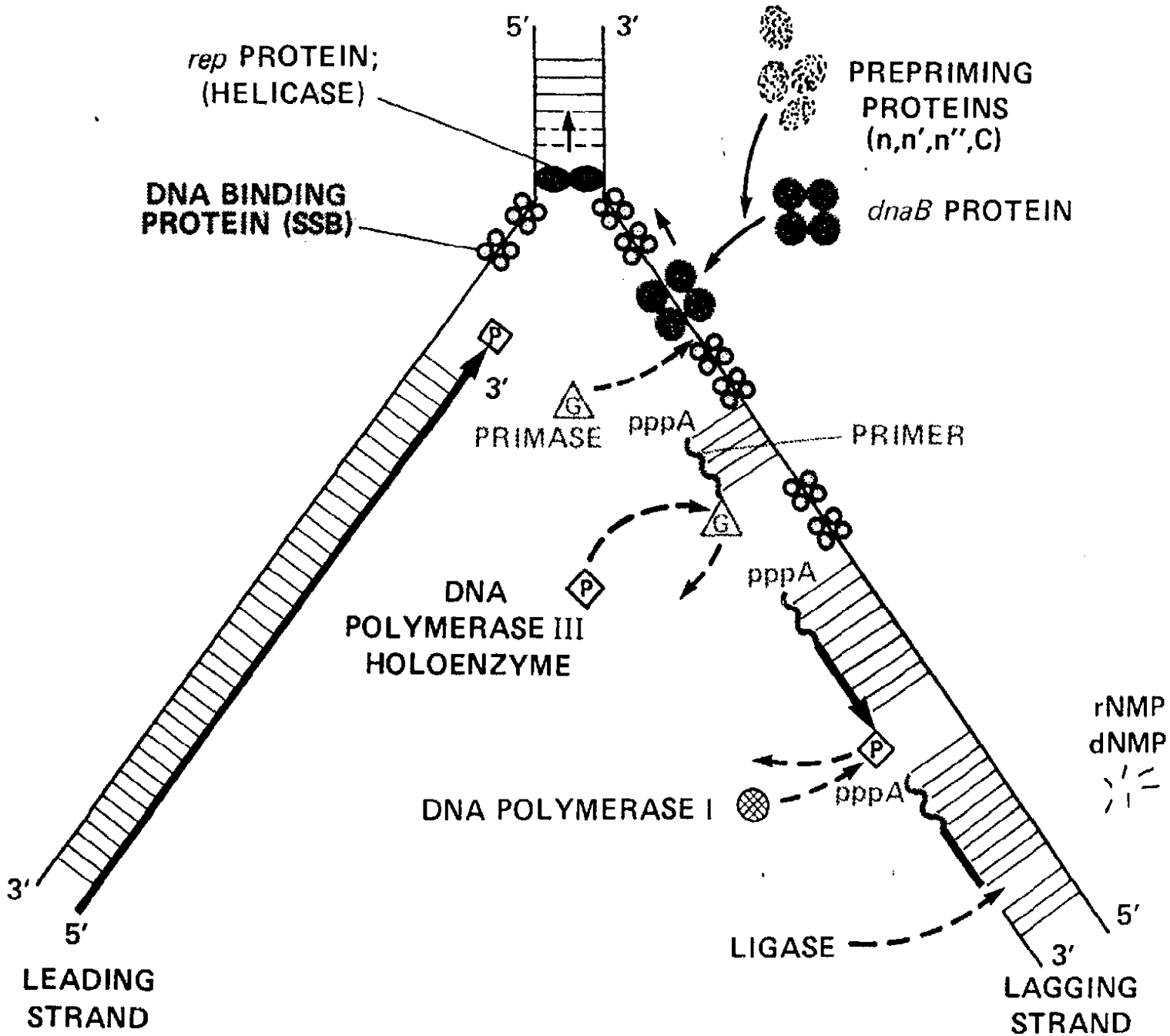
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 35- THE REPLICATION FORK HAS A COMPLEX ASSEMBLY OF ENZYMES.



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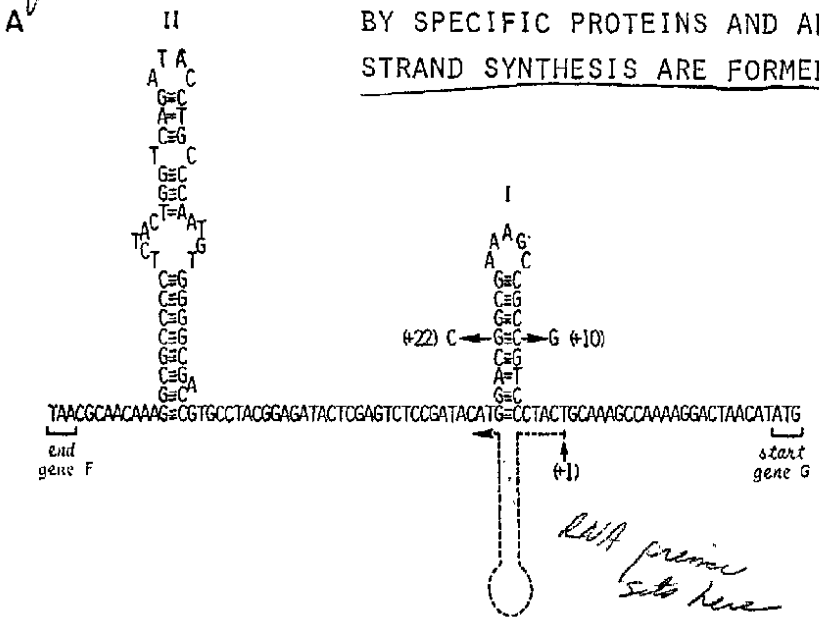
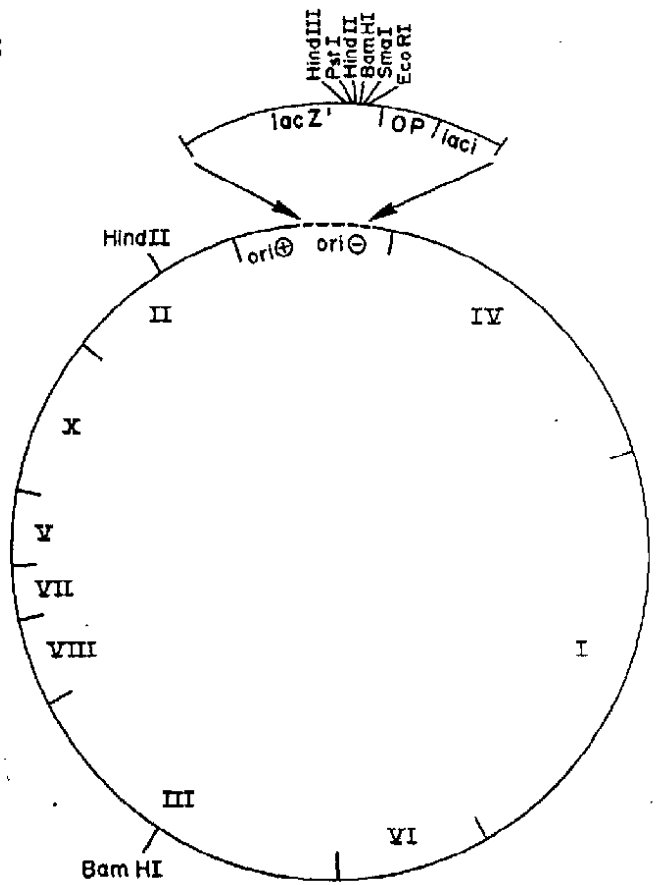
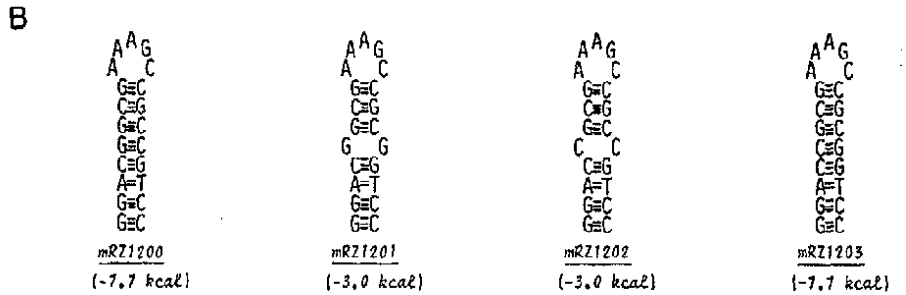
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Recognition 3° structure is also released

FIG 36- ORIGINS OF DNA REPLICATION ARE COMPLEX UNIQUE SEQUENCES RECOGNIZED BY SPECIFIC PROTEINS AND ARE THE LOCATION AT WHICH RNA PRIMERS FOR LEADING STRAND SYNTHESIS ARE FORMED.







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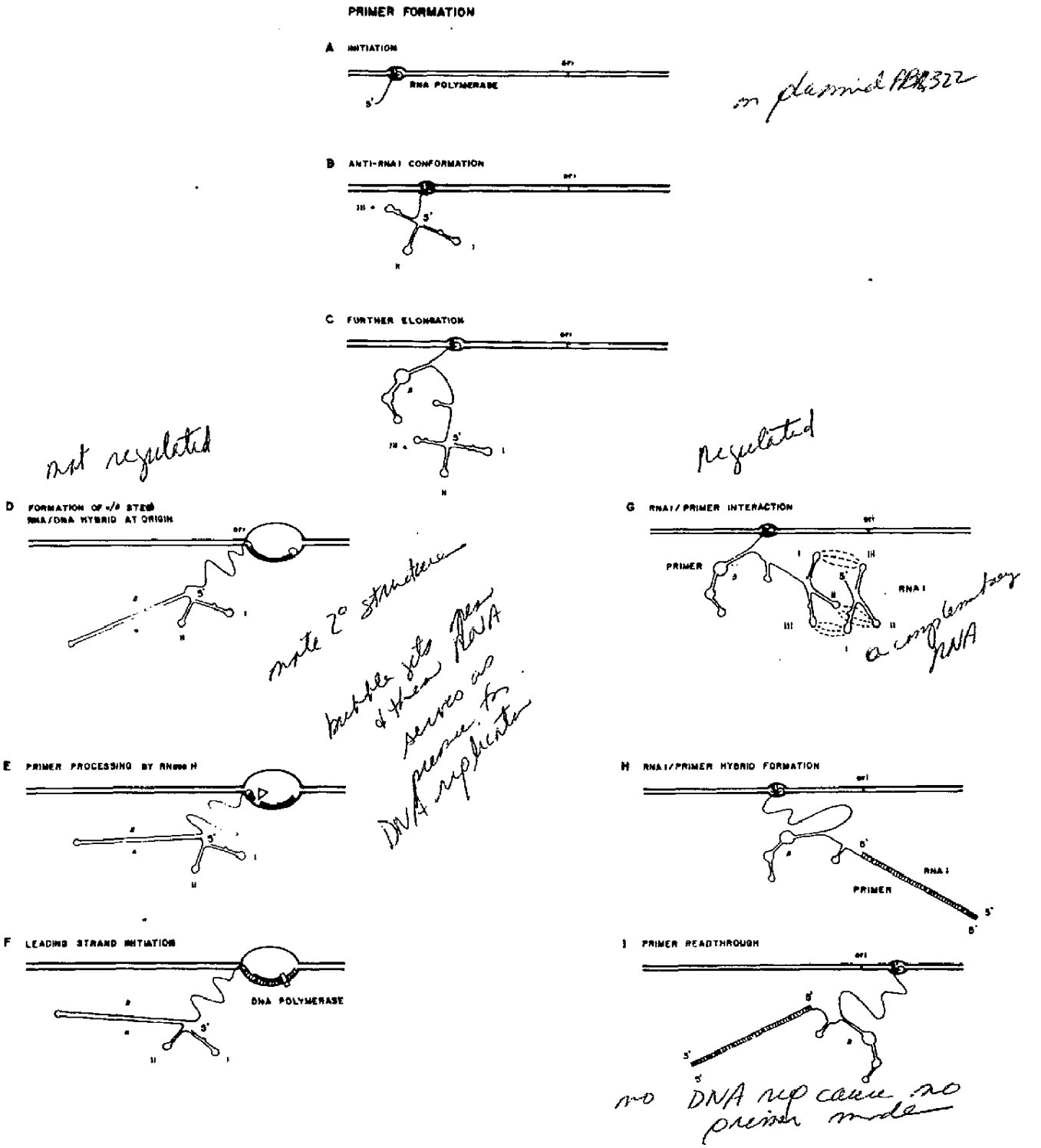
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

2° structure gives rise to "bubble" which can act as primer
DS RNA prevents "bubble" ⇒ no primer
this known empirically – reasons not known

FIG 37- THE INITIATION OF DNA REPLICATION IS OFTEN REGULATED BY REGULATING THE SYNTHESIS OF THE PRIMER.



on plasmid PBR322

not regulated

regulated

note 2° structure

bubble gets open & then RNA serves as primer for DNA replication

a complementary RNA

no DNA rep cause no primer made

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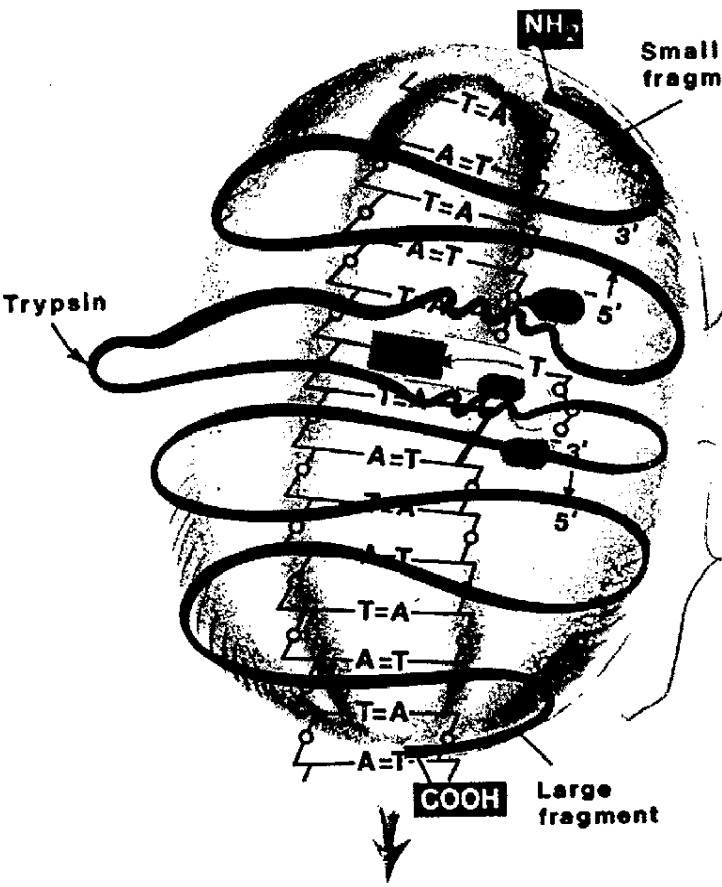
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

nuclease ≡ cuts phosphodiester bonds
exo ≡ works from an end
endo ≡ " in center

DNA Polymerase I from E. coli



5'–3' exonuclease activity (gets rid of RNA in leading strand in DNA replication) ①

FIG 38A- SOME DNA POLYMERASES HAVE OTHER ASSOCIATED ENZYME ACTIVITIES SUCH AS A 5' TO 3' EXONUCLEASE AND A 3' TO 5' PROOFREADING EXONUCLEASE.

② 3'→5' exo – can remove what's been put on

Polymerase activity (Klenow fragment)

Substrate for DNA polymerase is SS DNA molecule

FIG 38B- THE 3' TO 5' EXONUCLEASE.

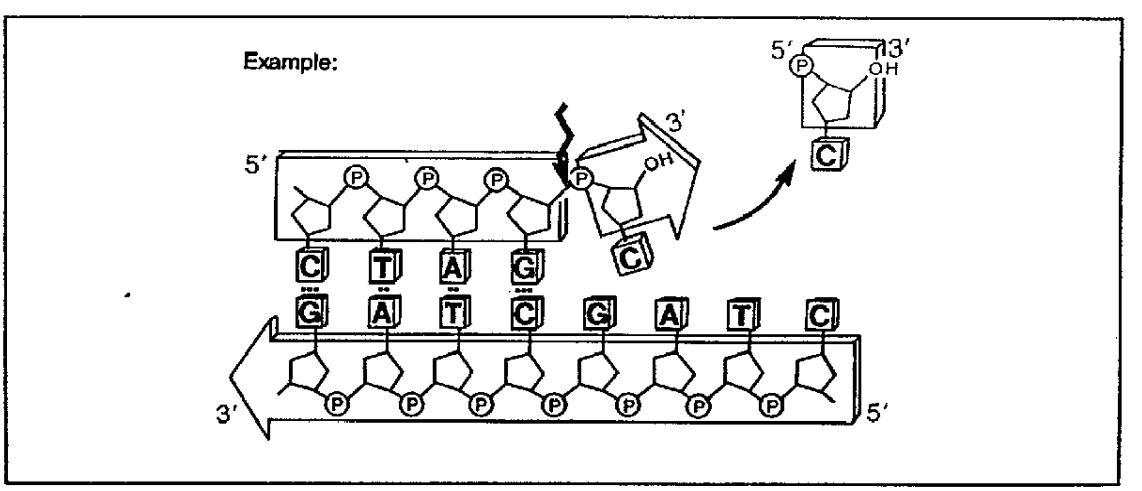
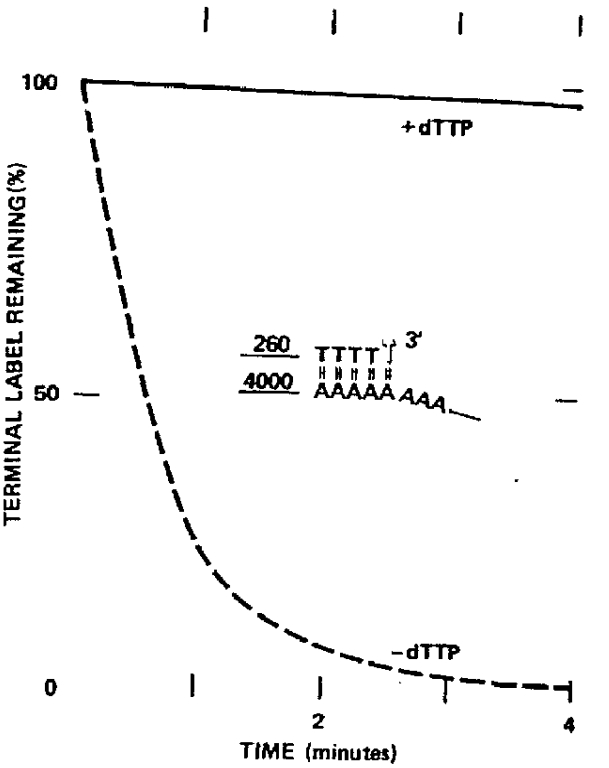


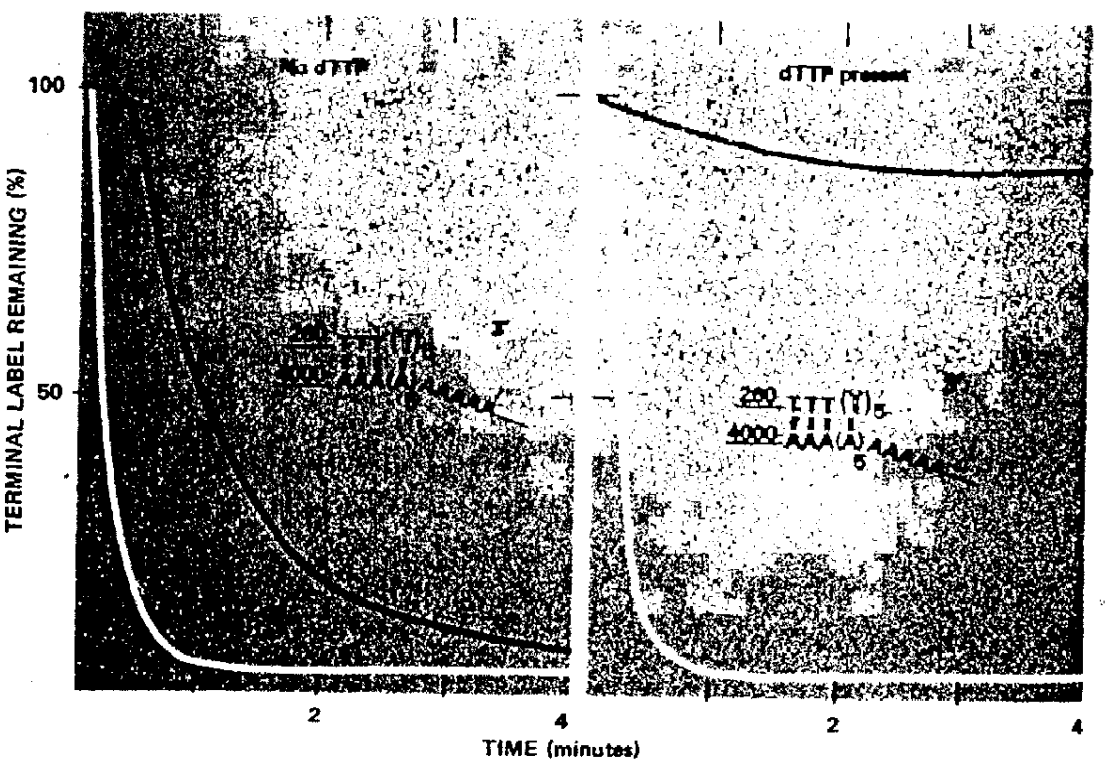
Figure 3.5.2 DNA polymerase 3'→5' exonuclease activity.

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

control the digestion by putting the substrate in first

FIG 39- THE EDITING FUNCTION OF THE 3' TO 5' EXONUCLEASE.





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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

RNA-dependent DNA Polymerase (reverse transcriptase) — useful for making cDNA

(avian myeloblastosis virus)

The enzyme consists of two polypeptides (one of which carries both a 5'→3' polymerase activity and a processive 5' and 3' riboexonuclease that is specific for RNA•DNA hybrids (Verma 1977).

Activity	Reaction	Template/primer or substrate
5'→3' DNA polymerase	DNA_{OH} or RNA_{OH} + ndNTP, Mg^{++} → $DNA\text{-}(pdN)_n$ + nPPi or $RNA\text{-}(pdN)_n$ + nPPi	RNA or DNA template RNA or DNA primer with $3'_{OH}$
5'→3' Exoribonuclease 3'→5' Exoribonuclease (RNase H)	RNA/DNA → DNA + oligoribonucleotides	specifically degrades RNA in a DNA•RNA hybrid by a processive mechanism

5' pT-pT-pT-pT-pT-OH 3'
...Ap-Ap-Ap-Ap-Ap-Up-Cp-Up-Gp-Up-Cp-Cp-Up-Ap 5'
(dATP, dCTP, dGTP, TTP, Mg^{++})
5' pT-pT-pT-pT-pT-pA-pG-pA-pC-pA-pG-pG-pA-pT-OH 3'
Ap-Ap-Ap-Ap-Ap-Up-Cp-Up-Gp-Up-Cp-Cp-Up-Ap 5'

FIG 40- REVERSE TRANSCRIPTASE CAN USE EITHER RNA OR DNA AS A TEMPLATE; IT MAKES DNA COPIES OF RNA.

Uses

1. Synthesis of cDNA for cloning (both first and second strands) or for hybridization probes.
2. Labeling the termini of DNA fragments with protruding 5' ends (filling reaction).

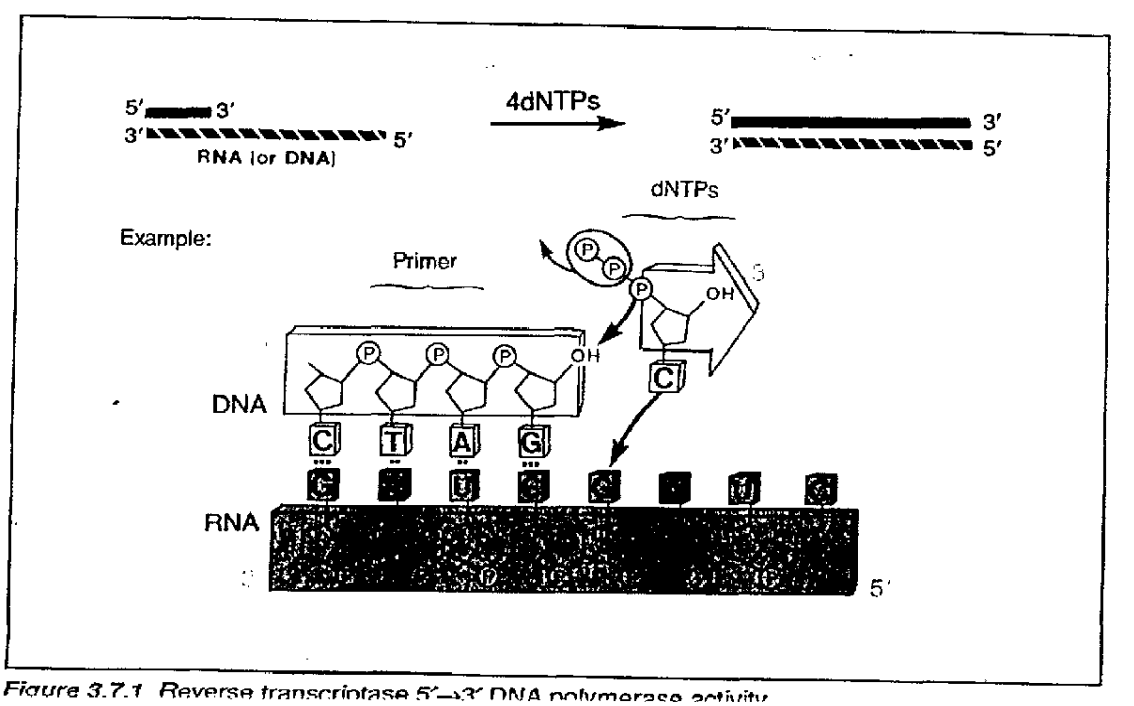

Figure 3.7.1 Reverse transcriptase 5'→3' DNA polymerase activity

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 41A- TERMINAL TRANSFERASE ADDS NUCLEOTIDES ONTO A 3' OH END WITHOUT THE USE OF A TEMPLATE.

Terminal Deoxynucleotidyl Transferase (terminal transferase)

(calf thymus)

The enzyme catalyzes the addition of deoxynucleotides to the 3'-OH end of DNA molecules (Bollum 1974).

Activity	Reaction	Template/primer or substrate
Terminal transferase	ss DNA_{OH} + ndNTP $\xrightarrow{Mg^{++}}$ DNA-$(pdN)_n$ + nPPi	single-stranded DNA with a 3'-OH terminus (or double-stranded DNA with a protruding 3'-OH terminus).
Terminal transferase	ds DNA_{OH} + ndNTP $\xrightarrow{Co^{++}}$ DNA-$(pdN)_n$ + nPPi	blunt-ended, double-stranded DNA or DNA with a recessed 3'-OH terminus serves as template if Co^{++} is supplied as cofactor (Roychoudhury et al. 1976).

Uses

1. Adding complementary homopolymer tails to vector and cDNA (see chapter 7).
2. Labeling the 3' ends of DNA fragments with a ^{32}P-labeled-3'-deoxynucleoside (Tu and Cohen 1980) or a 3'-ribonucleoside (Wu et al. 1976). For labeling with ribonucleosides, $[\alpha\text{-}^{32}P]$rNTP is used, followed by treatment with alkali.

FIG 41B- ALKALINE PHOSPHATASE REMOVES TERMINAL 5' PHOSPHATE GROUP.

Alkaline Phosphatase

(bacteria [BAP] ; calf intestine [CAP])

CIP

The enzyme catalyzes the removal of 5'-phosphate residues from DNA and RNA, ribo- and deoxyribonucleoside triphosphates.

Activity	Reaction	Substrates
Phosphatase	5' pDNA or 5' pRNA $\longrightarrow$ 5' $_{OH}$-DNA or 5' $_{OH}$-RNA	single- or double-stranded DNA and RNA; ribo- and deoxyribotriphosphates

Uses

1. To remove 5' phosphates from DNA or RNA prior to labeling the 5' end with ^{32}P.
2. To remove 5' phosphates from fragments of DNA in order to prevent self-ligation.

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

T4 = virus subject of early enzymological research

T4 Polynucleotide Kinase

(T4-infected E.coli)

The enzyme catalyzes the transfer of the γ-phosphate of ATP to a 5'-OH terminus in DNA or RNA (Richardson 1971).

Activity

Kinase (forward reaction)

Reaction

$DNA_{OH}^{5'}$ or $RNA_{OH}^{5'}$ + [γ-^{32}P]ATP —(Mg++, DTT)→ $[^{32}P]$DNA 5' or $[^{32}P]$RNA 5' + ADP

Substrate

single or double-stranded DNA with 5'-OH terminus

For example:

$A\text{-}P\text{-}P\text{-}P^{32}$ + $^{5'OH}C_p\text{-}G_p\text{-}C_p\ldots$

(DTT, Mg++)

$^{5'}{}^{32}p\text{-}C_p\text{-}G_p\text{-}C_p\ldots$ + A-P-P

FIG 42A- POLYNUCLEOTIDE KINASE ADDS A PHOSPHATE GROUP ONTO A 5' OH END; IT CAN BE USED TO RADIOLABEL 5' ENDS.

Kinase (exchange reaction)

$A\text{-}P\text{-}P\text{-}P^{32}$ + excess ADP + $^{5'}p\text{-}C_p\text{-}G_p\text{-}C_p\ldots$

(DTT, Mg++)

$^{32}p\text{-}C_p\text{-}G_p\text{-}C\ldots$ + A-P-P + A-P-P-P

single or double-stranded DNA with 5'-P terminus

The excess ADP drives the exchange reaction, causing polynucleotide kinase to transfer the terminal 5'-phosphate from DNA to ADP. The DNA is then rephosphorylated by transfer of the labeled γ-phosphate in the [α-^{32}P]ATP (Berkner and Folk 1977).

Uses

1. Labeling 5' termini in DNA for sequencing by the Maxam-Gilbert technique (Maxam and Gilbert 1977).
2. Phosphorylating synthetic linkers and other fragments of DNA lacking 5'-P termini prior to ligation.

Nuclease Bal31

(Alteromonas espejiana Bal31)

The enzyme carries two activities (Lau and Gray 1979): (1) a highly specific, single-stranded endodeoxyribonuclease and exonuclease that catalyzes the removal of small oligonucleotides or mononucleotides from both 5' and 3' termini of double-stranded DNA (both strands of DNA are degraded at approximately the same rate); and (2) a single-strand-specific endonuclease similar to nuclease S1.

FIG 42B- BAL31 IS A DOUBLE STRANDED EXONUCLEASE USED FOR CREATING DELETIONS.

will work on both ends

Activity

Endodeoxyribonuclease/ exonuclease

Reaction

5' 3' / 3' 5' —(Ca++)→ 5' 3' / 3' 5' + oligo- and mononucleotides

Substrate

double-stranded DNA with blunt or protruding termini

Single-stranded endonuclease

ss DNA —(Ca++)→ oligo- and mononucleotides

single-stranded DNA

Uses

1. Removing nucleotides from the termini of double-stranded DNA. The resulting molecules are shortened and can be joined to synthetic linkers or to vectors by T4 polynucleotide ligase.
2. Mapping restriction sites in DNA (Legerski et al. 1978).

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Nuclease S1

(Aspergillus oryzae)

The enzyme degrades single-stranded DNA (Vogt 1973) to yield 5' phosphoryl mono- or oligonucleotides. Double-stranded DNA, double-stranded RNA, and DNA•RNA hybrids are relatively resistant to the enzyme. However, duplex nucleic acids are digested completely by S1 if they are exposed to very large amounts of the enzyme.

Activity	Reaction	Substrate
Single-stranded-specific nuclease	ssDNA or ssRNA ↓ pH4.5 Zn++ ↓ $5'_{pdN}$ or $5'_{prN}$	single-stranded DNA or RNA; more active on DNA than RNA

Moderate amounts of the enzyme will cleave duplex nucleic acids at nicks or small gaps (Kroeker and Kawalski 1978).
For example:

nicked dsDNA
↓ pH4.5 Zn++
+

FIG 43A- S1 NUCLEASE SPECIFICALLY ATTACKS SINGLE STRANDED POLYNUCLEOTIDES.

green

part of longer strand under brace will be degraded by S1 - since it looks to awhile like SS!

Uses

1. Analyzing the structure of DNA•RNA hybrids (Berk and Sharp 1978). For a detailed description of this technique, see Favaloro et al. (1980)
2. Removing single-stranded tails from DNA fragments to produce blunt ends.
3. Opening the hairpin loop generated during synthesis of ds-cDNA.

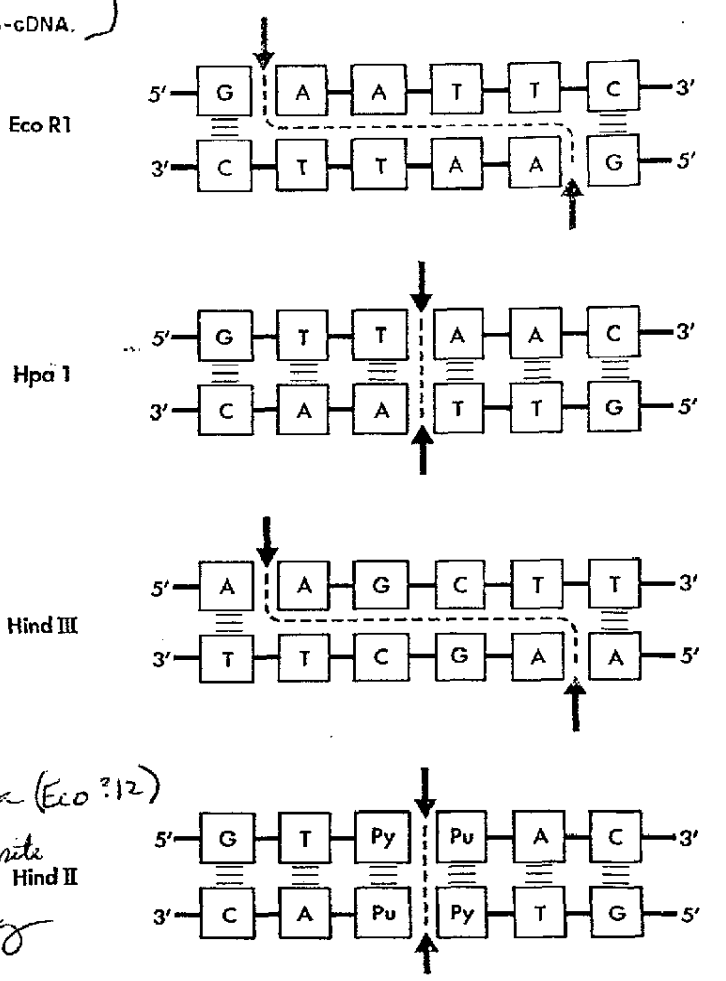

FIG 43B- RESTRICTION ENZYMES (TYPE II) ARE SEQUENCE SPECIFIC ENDONUCLEASES; SOME MAKE STAGGERED CUTS (STICKY ENDS), SOME MAKE BLUNT CUTS.

Modification enzyme to Eco R1 puts methyls on adenines

3 classes of restriction enzymes

I bends to specific sequence & goes a (Eco ?12) random distance down to cut

II bind & cut at same site - cut w/i the site

III bends & cuts a regular distance away from binding site

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Note: ① Size of target sequence varies
② "Dyne" of cutting varies – always give 5' PDY = + 3'OH
some blunt; some staggered

FIG 44- MORE RESTRICITION ENZYMES.

Some restriction endonucleases and their cleavage sites

Microorganism	Name of enzyme	Target sequence and cleavage sites
Generates cohesive ends		
Escherichia coli RY13	EcoRI	G↓A A|T T C C T T|A A↑G
Bacillus amyloliquefaciens H	BamHI	G↓G A|T C C C C T|A G↑G
Bacillus globigii	BglII	A↓G A|T C T T C T|A G↑A
Haemophilus aegyptius	HaeII	Pu G C|G C↓Py Py↑C G|C G Pu
Haemophilus influenza R_d	HindIII	A↓A G|C T T T T C|G A↑A
Providencia stuartii	PstI	C T G|C A↓G G↑A C|G T C
Streptomyces albus G	SalI	G↓T C|G A C C A G|C T↑G
Xanthomonas badrii	XbaI	T↓C T|A G A A G A|T C↑T
Thermus aquaticus	TaqI	T↓C|G A A G|C↑T
Generates flush ends		
Brevibacterium albidum	BalI	T G G↓|C C A A C C|G G T
Haemophilus aegyptius	HaeI	(A/T) G G↓|C C (T/A) (A/T) C C↑|G G (T/A) → AGGCCT, AGGCCA, TGGCCT, TGGCCA, etc.
Serratia marcescens	SmaI	C C C↓|G G G G G G↑|C C C

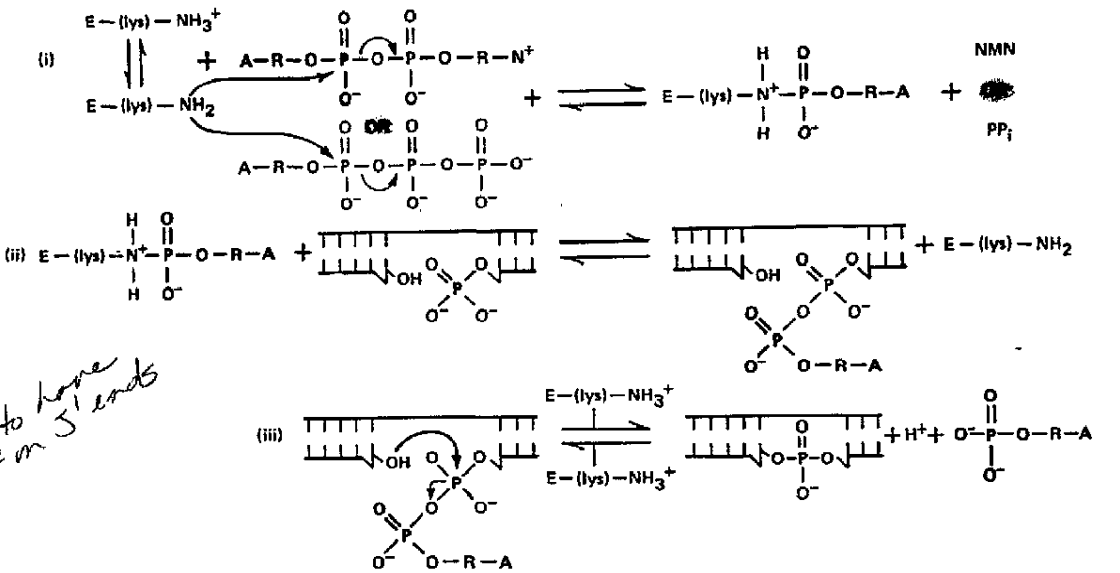
Note: The vertical dashed line indicates the axis of dyad symmetry in each sequence. Arrows indicate the sites of cutting. The enzyme TaqI yields cohesive ends consisting of two nucleotides, whereas the cohesive ends produced by the other enzymes contain four nucleotides. The enzyme HaeI recognizes the sequence GGCC whether the adjacent base pair is A·T or T·A, as long as dyad symmetry is retained. Pu and Py refer to any purine and pyrimidine, respectively.

Wisconsin is capital of Restriction Enzymes DNA*; Genetics Computer Group

PM3001193121

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 45- LIGASE LINKS A 5' PO_4 TO A 3' OH GROUP TO FORM A PHOSPHODIESTER BOND IN DNA. LIGASE WORKS MOST EFFECTIVELY WHEN ENDS ARE HELD TOGETHER THROUGH HYDROGEN BONDING.



have to have PO_4 ≡ on 5' ends

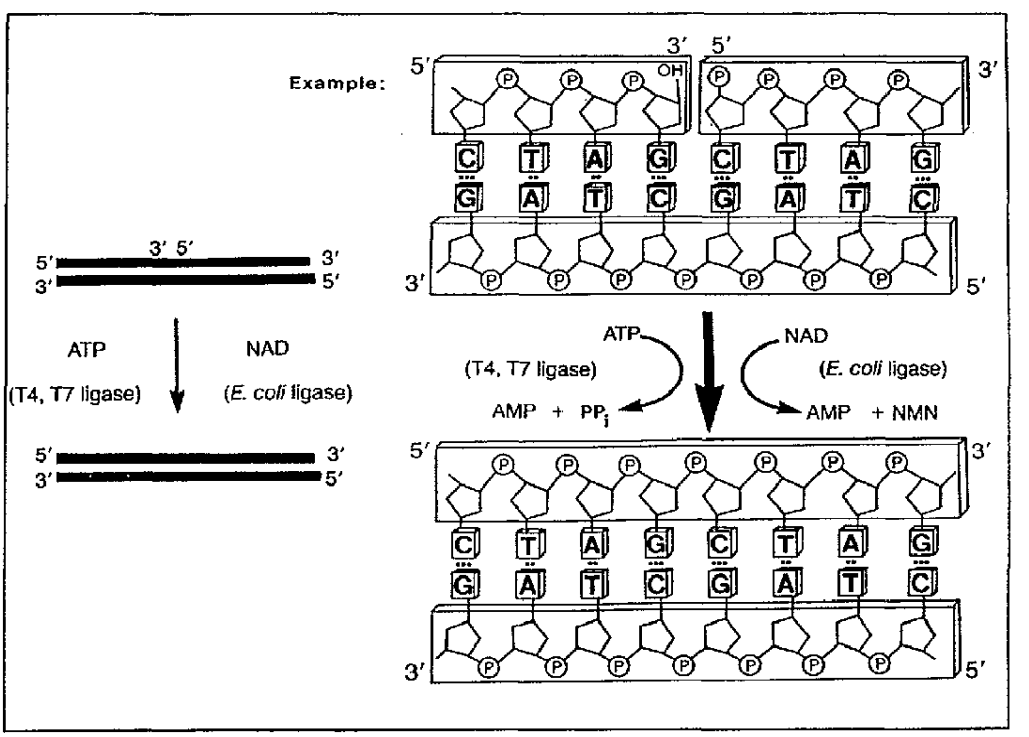


Figure 3.14.1 DNA ligase activity at a nick.

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 46- LIGASE WILL ALSO LINK DNA MOLECULES WITH COMPLEMENTARY OR COHESIVE ENDS, AND, AT A LOWER EFFICIENCY, BLUNT OF FLUSH ENDED DNA MOLECULES.

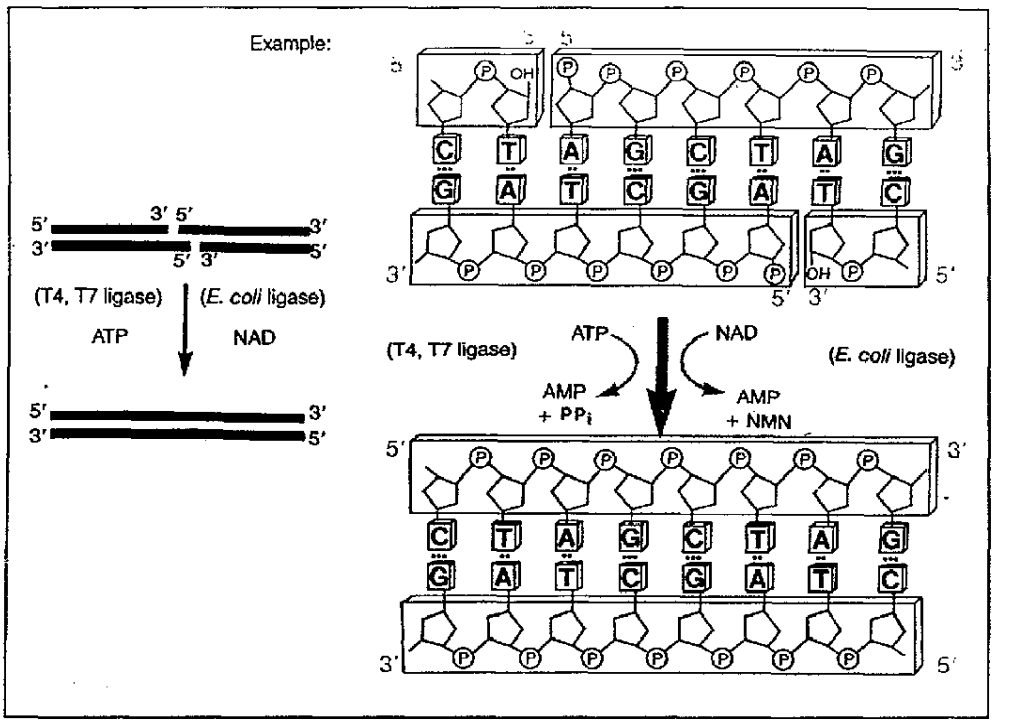

Figure 3.14.3 DNA ligase activity at cohesive ends.

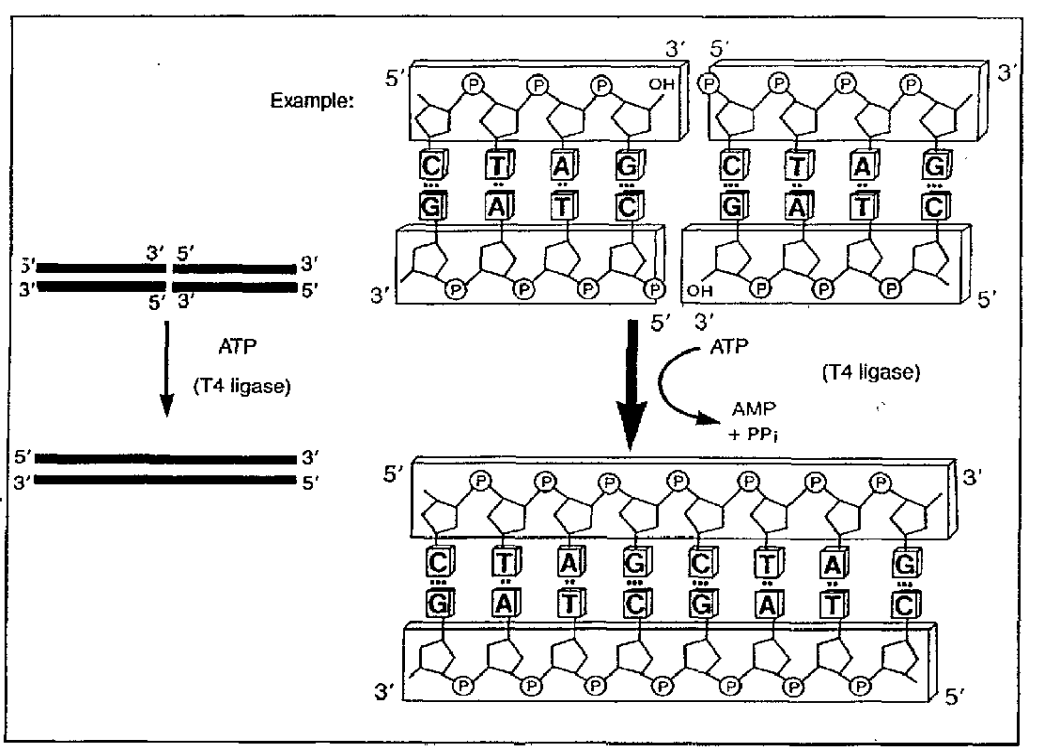

Figure 3.14.2 DNA ligase activity at blunt ends.

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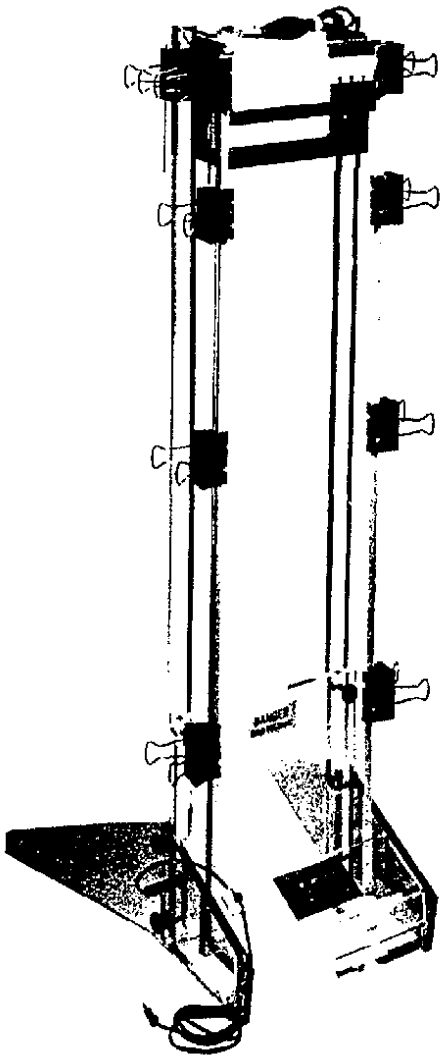
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

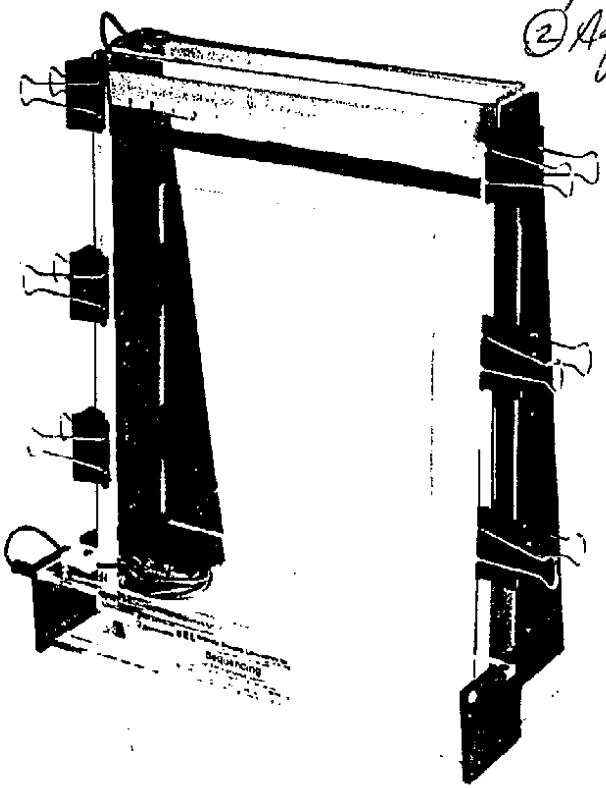
FIG 47- POLYACRYLAMIDE GEL ELECTROPHORESIS IS USED TO SEPARATE DNA MOLECULES OF DIFFERENT SIZES (OR CONFORMATIONS).

Model S1



BRL has designed, produced and laboratory-tested two Gel Electrophoresis Systems to meet your complete apparatus needs for nucleic acid sequence analysis. Both systems feature BRL's unique Silicon and Neoprene gasket system which provides a fluid tight seal, direct buffer gel contact and eliminates the need for fragile, expensive, notched glass plates. Each model also comes complete and ready to use with glass plates, power cords, clips, combs and spacer set in the thickness you select.

Model S0



Features

- The ultimate in format flexibility; fully adjustable for any gel length from 30 cm to 85 cm.
- 17 cm gel width accommodates either the 12 or 18 sample well comb.
- Free standing gel sandwich eliminates temperature "hot spots."

Features

- 34 cm x 40 cm slab gel format, compatible with standard medical x-ray film.
- 16, 20 and 32 tooth combs and spacers in 0.4, 0.8 or 1.6 mm thickness.

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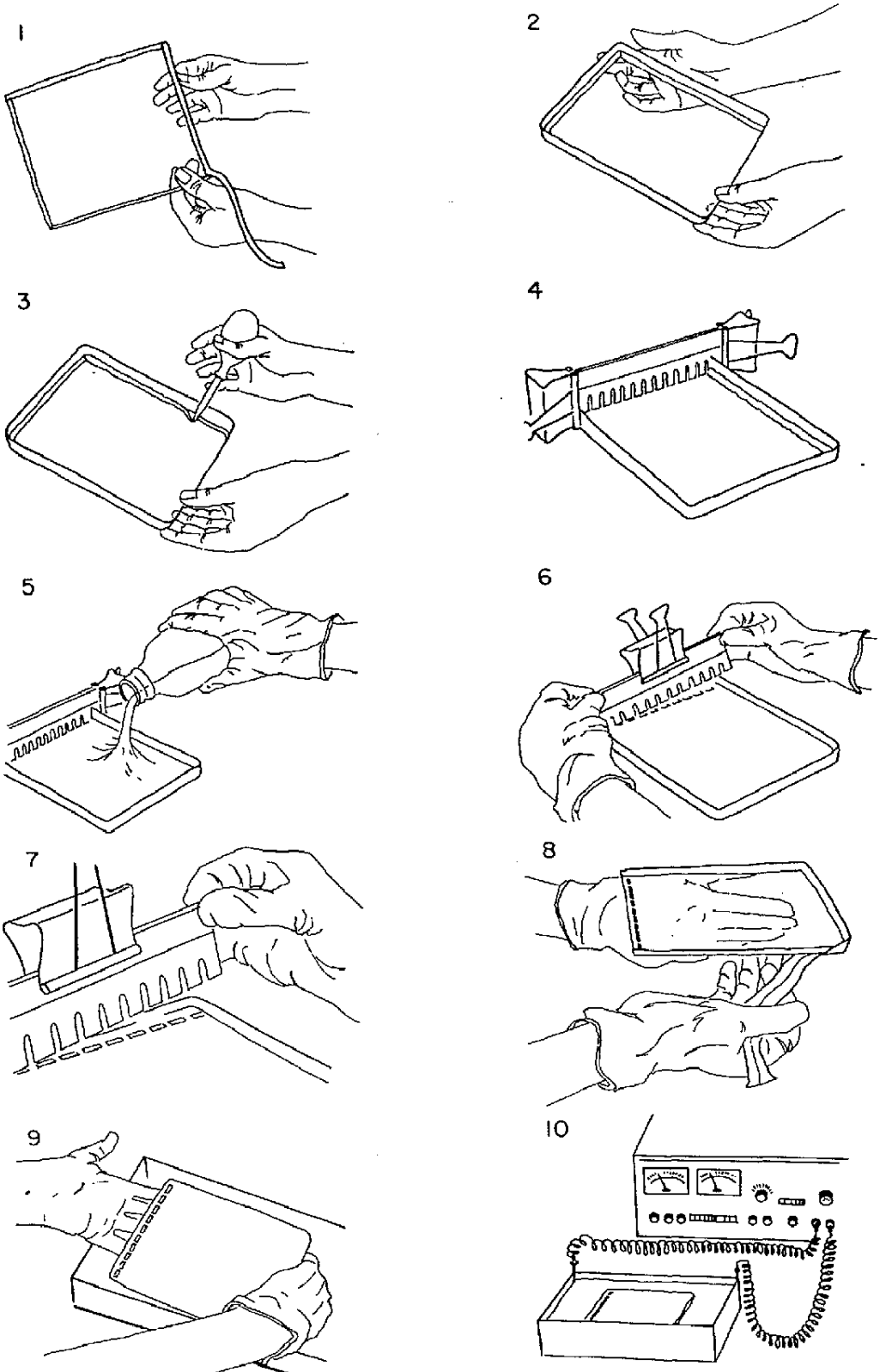
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 48- AGAROSE GEL ELECTROPHORESIS IS USED TO SEPARATE LARGER DNA MOLECULES.



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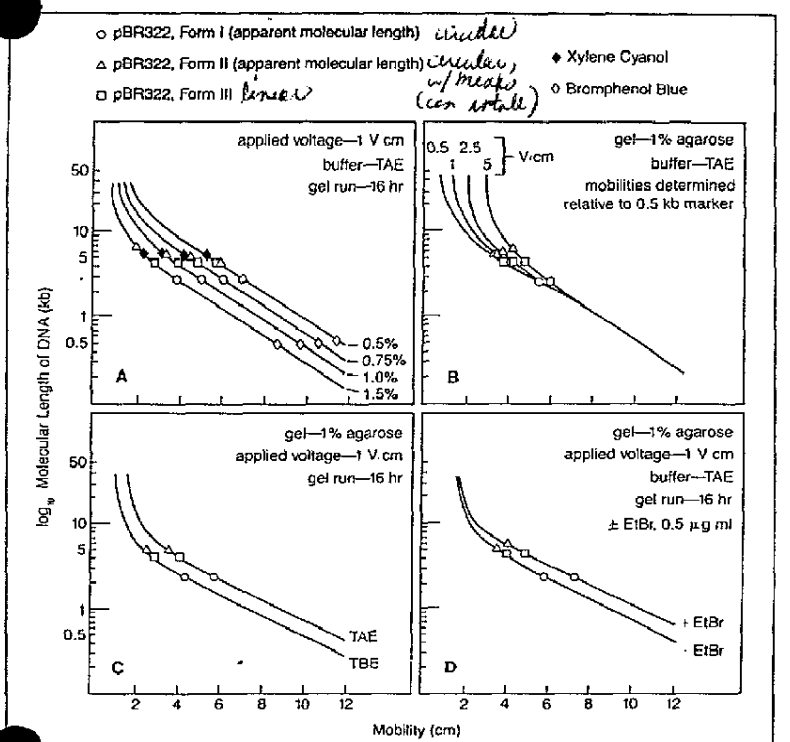
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 49a - A TYPICAL SEPARATION OF DIFFERENT LINEAR DNA MOLECULES ON AN AGAROSE GEL.

FIG 49b - ELECTROPHORETIC SEPARATION OF DUPLEX DNA MOLECULES FRACTIONATES THE MOLECULES ACCORDING TO THEIR FRICTIONAL COEFFICIENTS. THIS SEPARATION IS USUALLY INVERSELY RELATED TO THEIR CHAIN LENGTHS.

↑ Agarose [ ] ⇒ ↓ mobility for given MW

↑ Agarose [ ] ⇒ ↓ MW cut-off



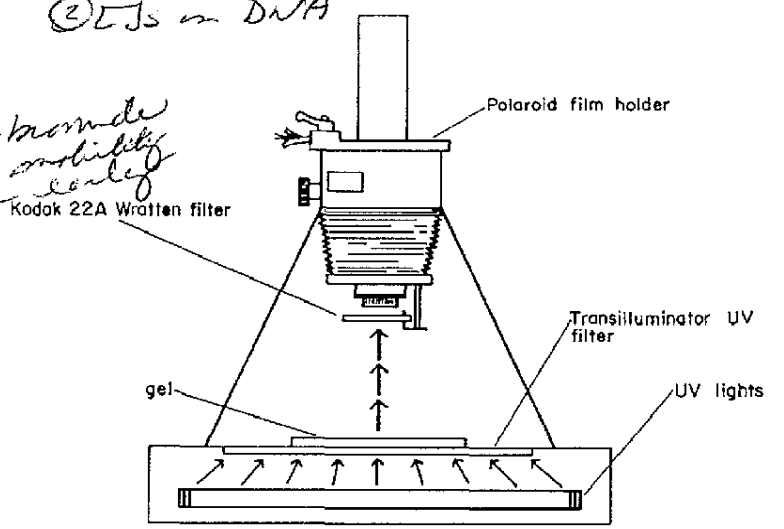
re 2.5.1 Effect of (A) agarose concentration, (B) applied voltage, (C) electrophoresis buffer, and (D) ethidium bromide on migration of DNA through agarose gels.

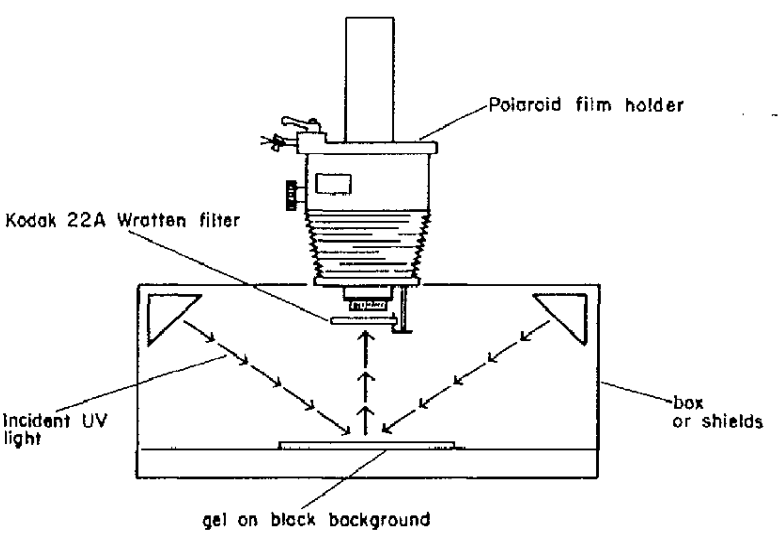
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 50 · DNA MOLECULES CAN BE VISUALIZED IN A GEL BY "STAINING" WITH ETHIDIUM BROMIDE FOLLOWED BY UV ILLUMINATION.

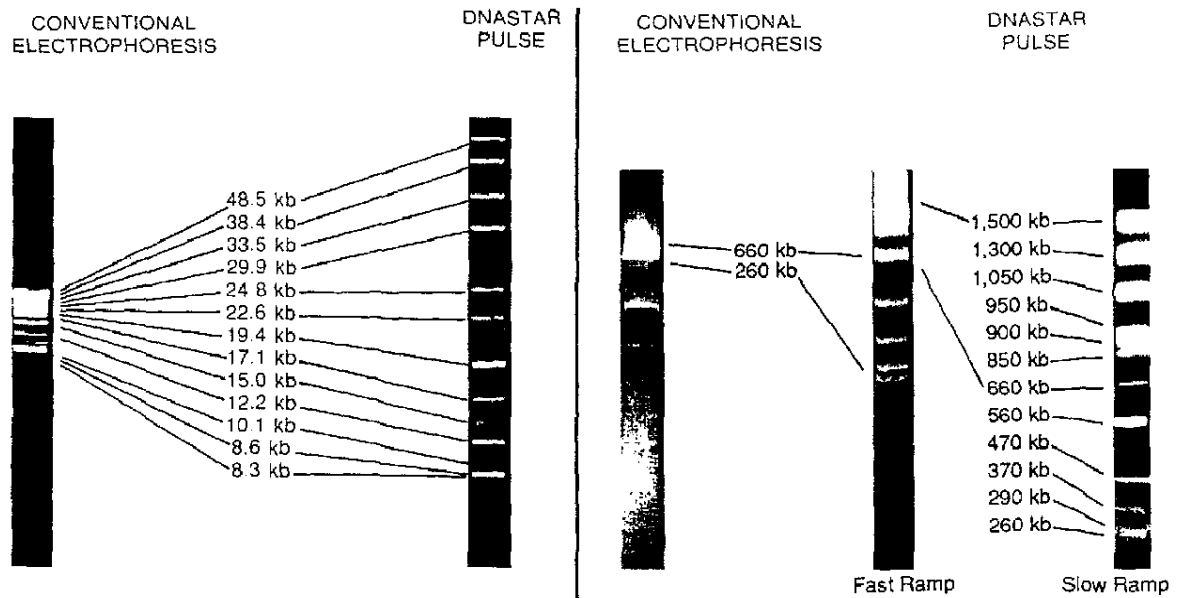
ethidium Br fluoresces
intercalates into DNA
① enhances fluorescence
② [ ]s in DNA

See 49b – ~~Ethr~~ Ethidium bromide will Δ mobility if added early





Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001



— Fig 2. Field Inversion Gel Electrophoresis is one of several techniques which permit separation of very large molecules presumably by fractionating according to how easily a molecule reorients itself when experiencing a reorienting electric field. —

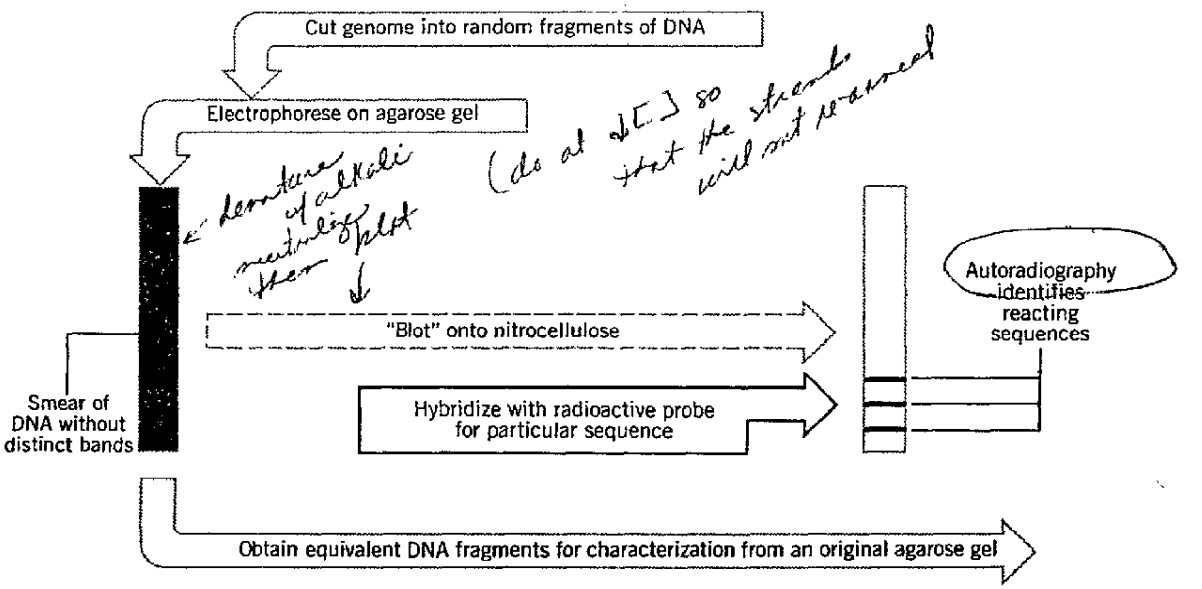
Needed to separate ≥ 20,000 bp species

can go $> 1 \times 10^6$ bp

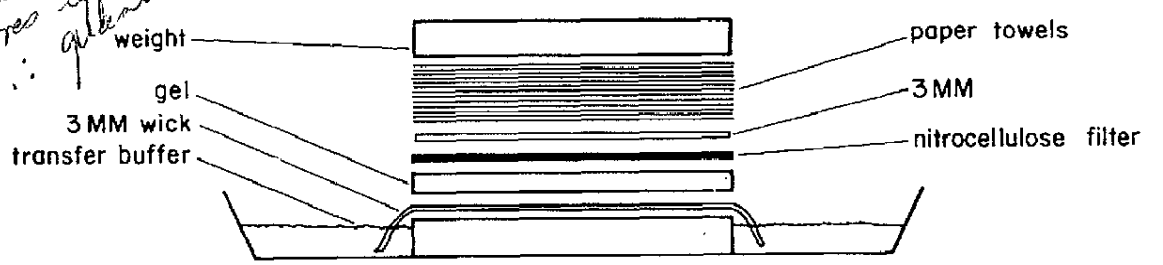
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

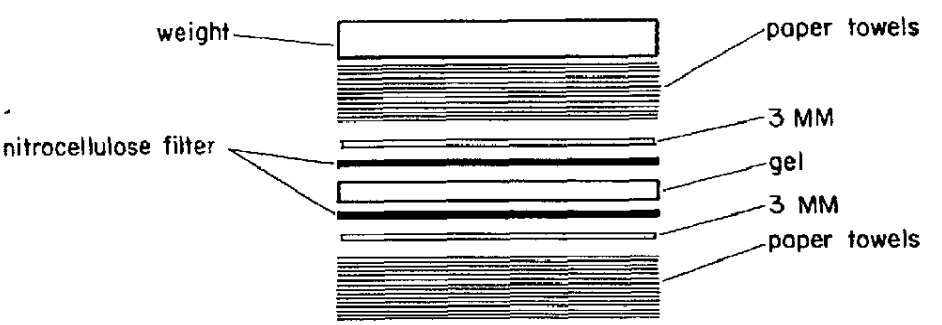
Southern is Dr. who died
Northern vs RNA
Western is protein — typically probed w/ Ab

FIG 51- SPECIFIC DNA MOLECULES CAN BE DETECTED IN A GEL BY A FILTER PAPER HYBRIDIZATION TECHNIQUE TERMED "SOUTHERN" BLOTTING.



electroblotting faster and more efficient ∴ quantitative aspects





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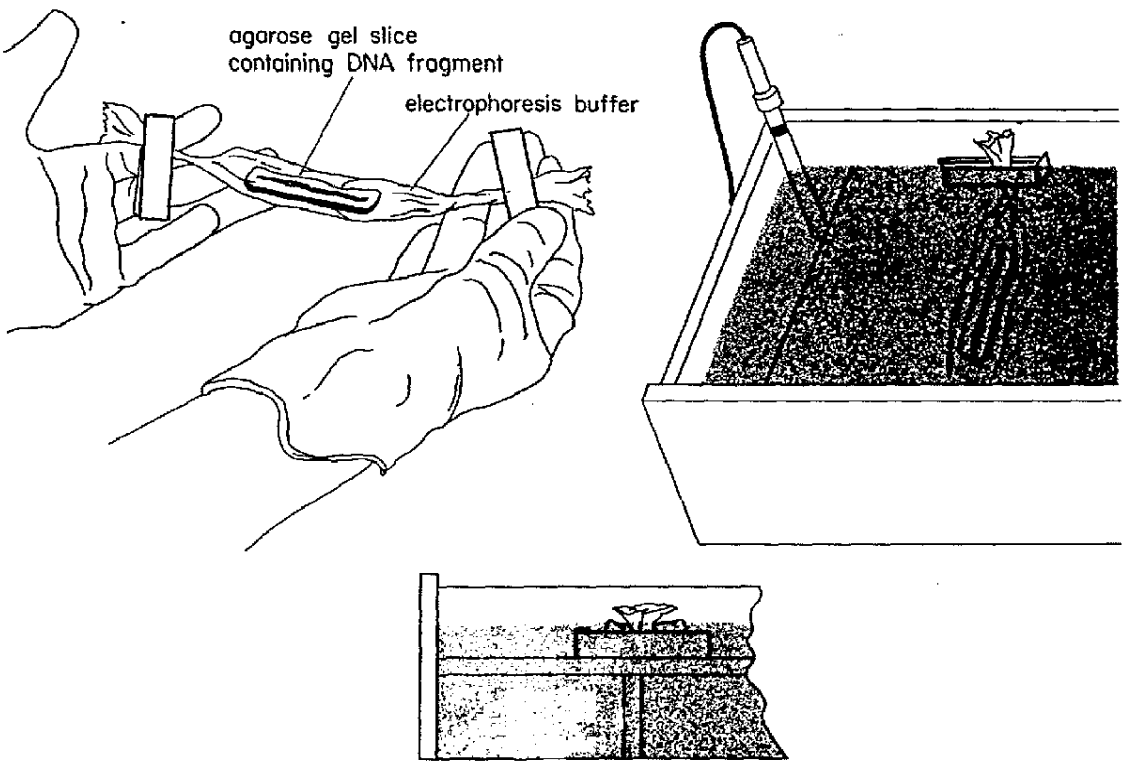
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 52- DNA MOLECULES CAN BE REMOVED FROM A GEL BY MANY TECHNIQUES INCLUDING ELECTROELUTION.



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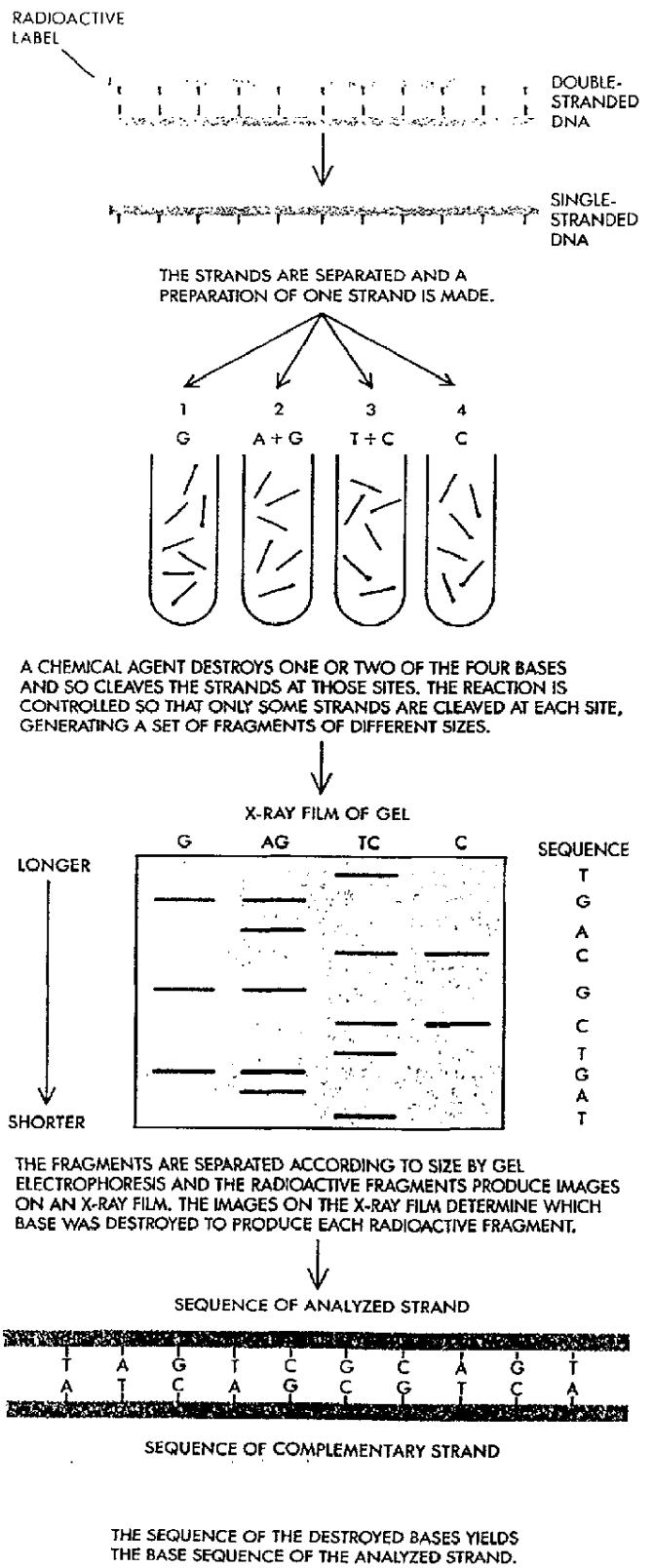
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Can get ~1000 bases/day after data have been checked, cleaned, etc.

FIG 53 - DNA SEQUENCE ANALYSIS BY THE MAXAM-GILBERT CHEMICAL CLEAVAGE TECHNIQUE.



Start of molecule w/ label at one end

make SS separate strands

Dimethyl sulfate will methylate guanine & yield preferentially to hydrazine cleavage

Different reagents for different bases

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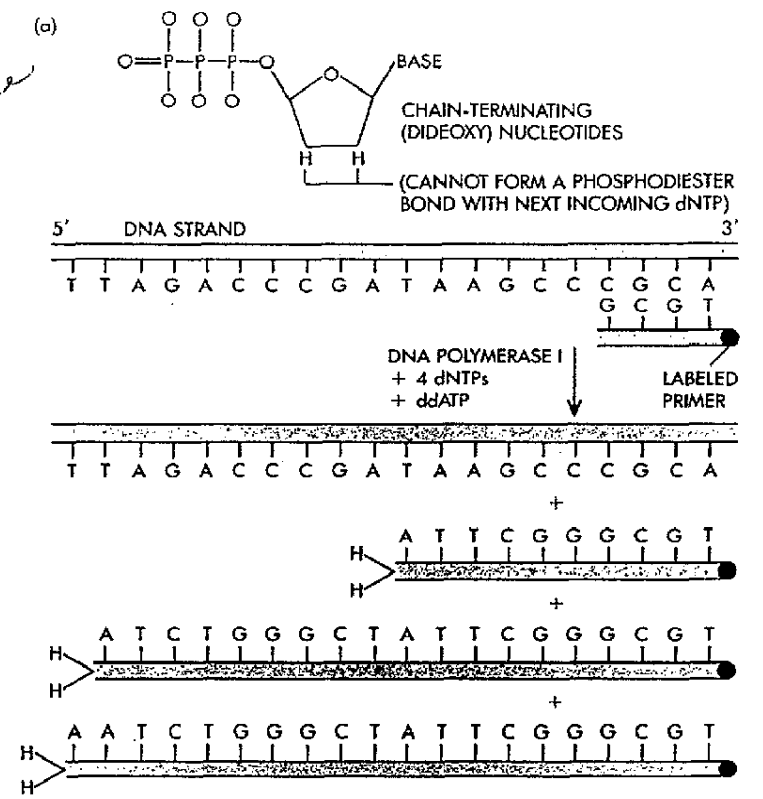
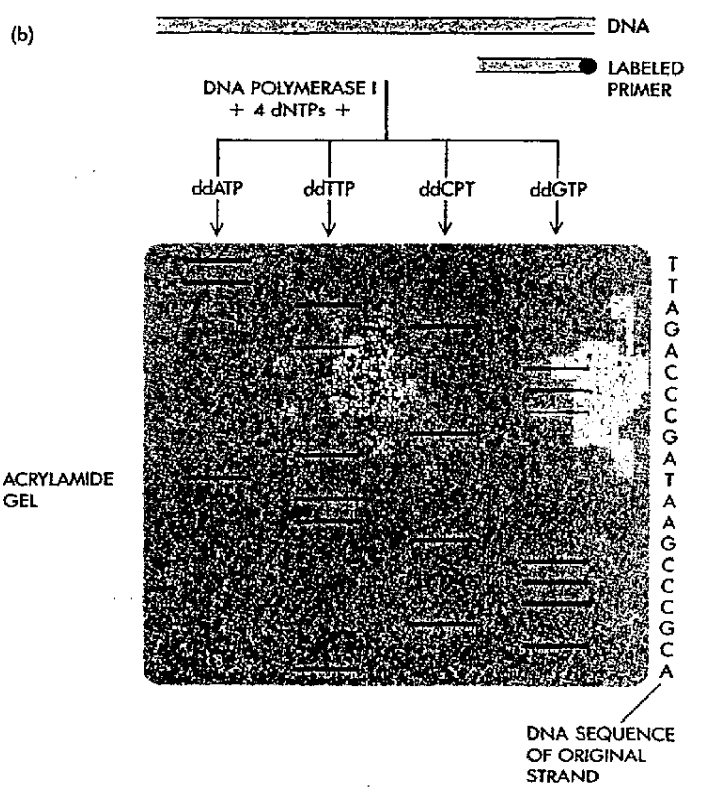
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Automated sequencers typically based on Sanger technique

FIG 54 - DNA SEQUENCE ANALYSIS BY THE SANGER DIDEOXY ENZYMATIC TECHNIQUE.

can use this technique w/ RNA if you use reverse transcriptase





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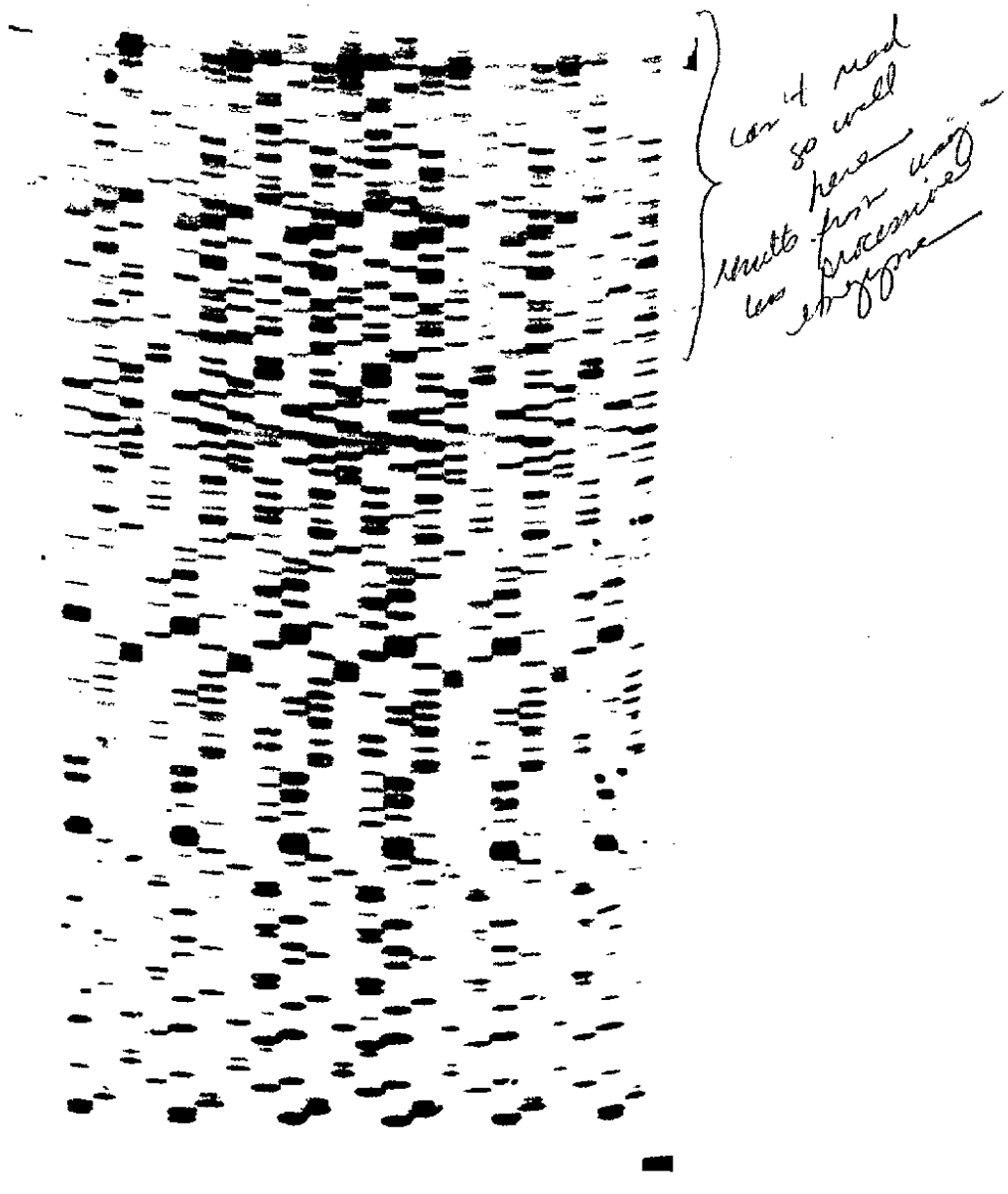
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 55- TYPICAL PAGE DNA SEQUENCE ANALYSIS.



Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

TAQ polymerase made this possible, since it was heat stable + more processive (relative to Klenow)

FIG 56- POLYMERASE CHAIN REACTION (PCR) TECHNOLOGY AMPLIFIES SELECTED DNA SEQUENCES THROUGH REPEATED ROUNDS OF SPECIFICALLY PRIMED DNA POLYMERIZATIONS.

makes one sequence @ what you know the ends

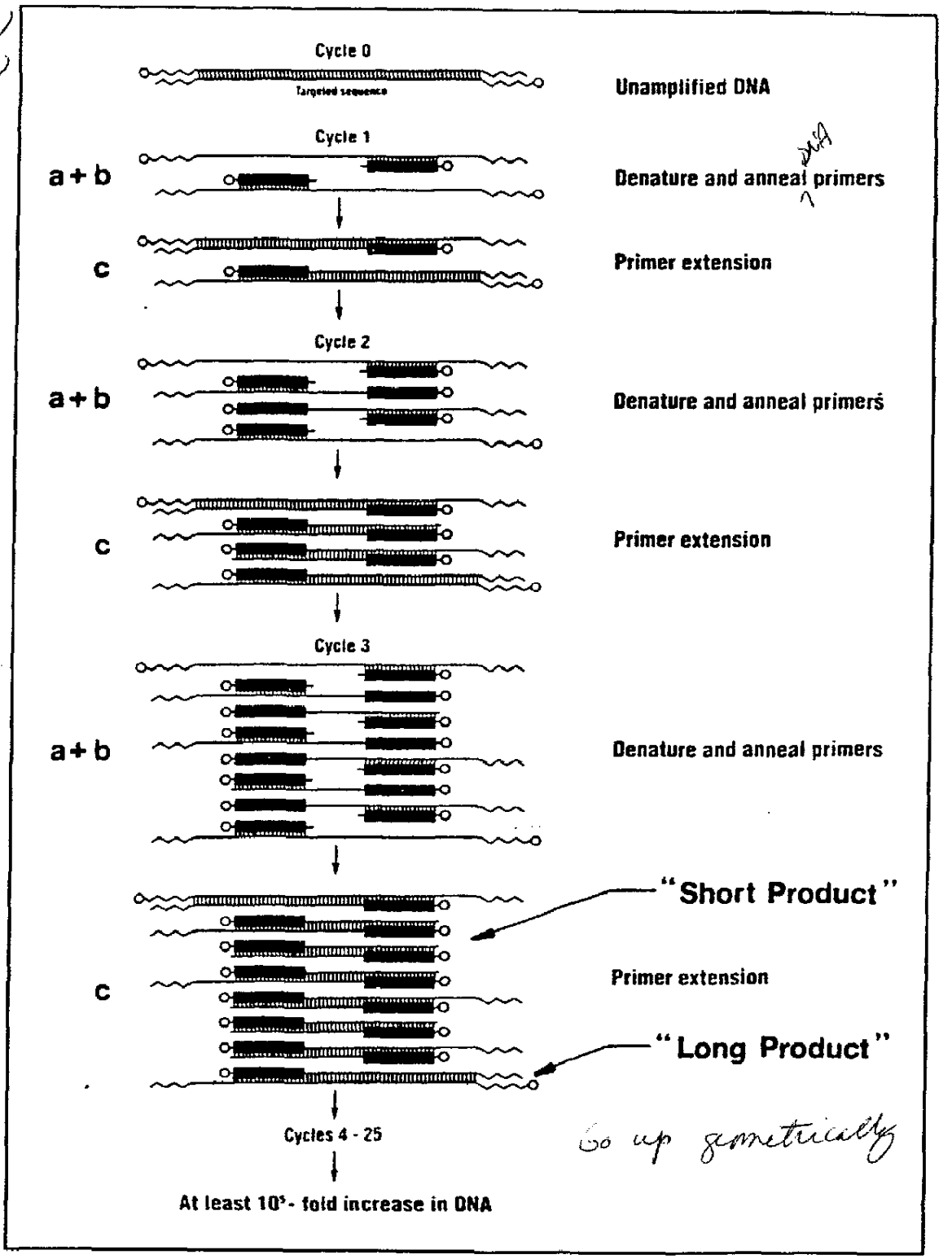
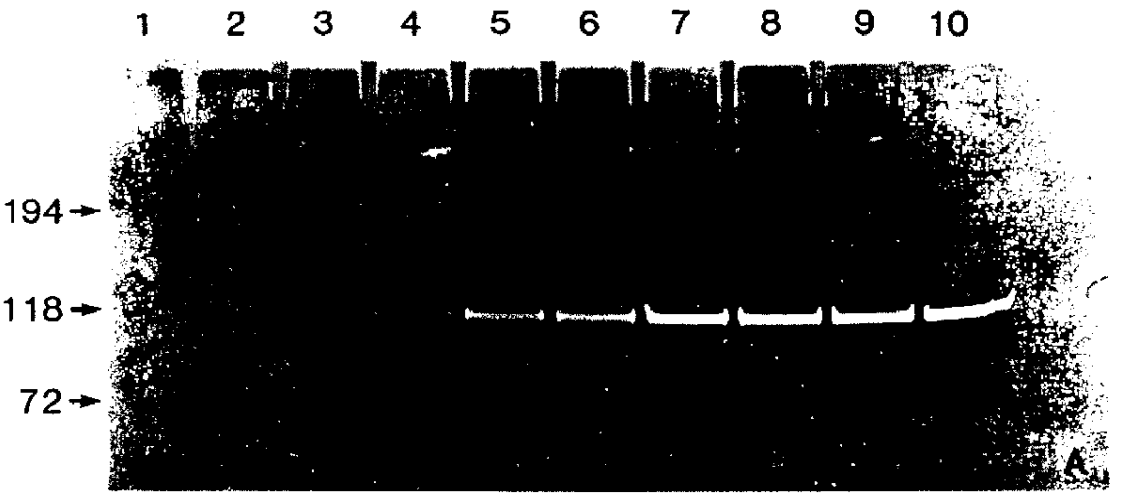
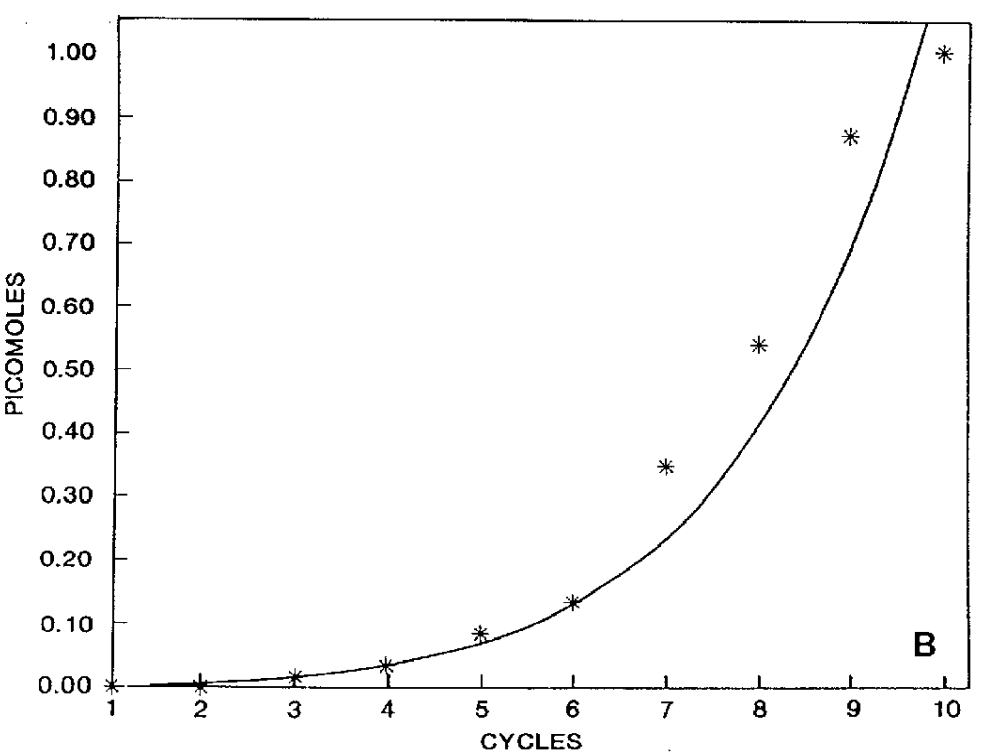


Figure 1. Polymerase chain reaction: Principles of the method.

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 57 - PCR AMPLIFICATION OF A SPECIFIC DNA SEQUENCE IS ILLUSTRATED.





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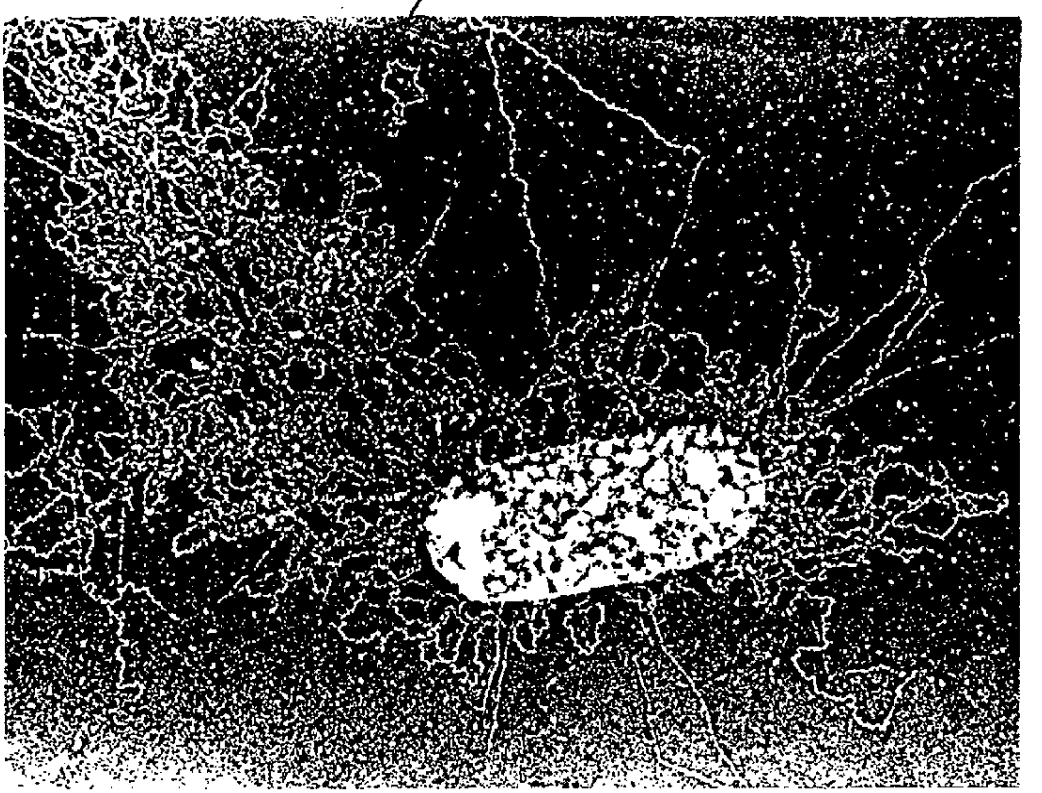
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 58- ELECTRON MICROGRAPH OF TWO TYPES OF DNA MOLECULES FOUND IN E. COLI THE SPAGETTI-LIKE CHROMOSOMAL DNA SHOWS THE SCIENTIFIC PROBLEM (THE ENORMOUS COMPLEXITY OF CHROMOSOMES), THE SECOND MOLECULE, A PLASMID, SUGGESTS A SOLUTION. PLASMIDS ARE MUCH SIMPLER AND, FOR SOME TYPES, MORE ABUNDANT.



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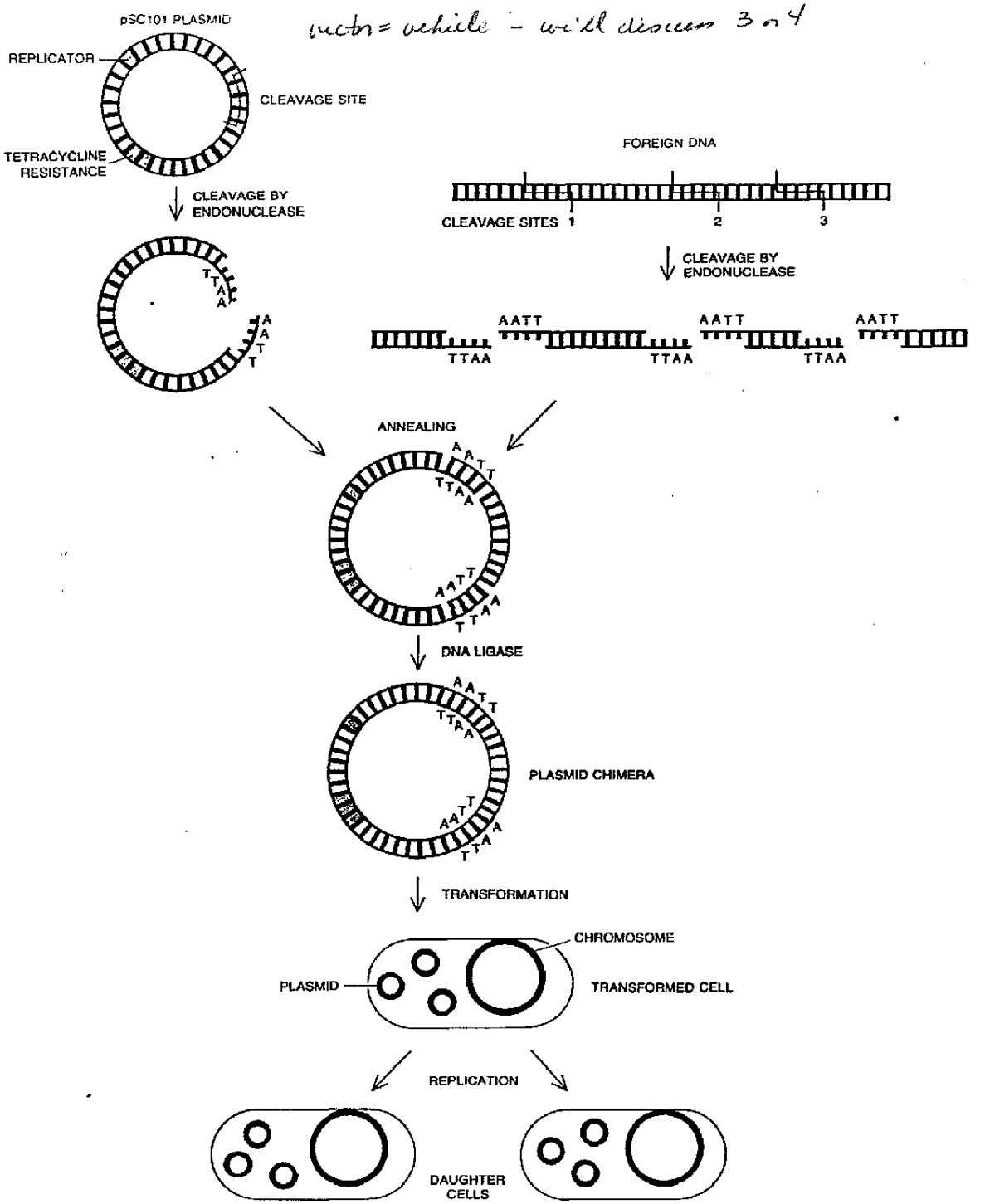
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

How will you know you've got the sequence you want out in the DNA?

FIG 59- BASIC CLONING STRATEGY; INSERT A SMALL DNA FRAGMENT FROM A COMPLEX OR RARE SOURCE INTO A SIMPLE, HIGH COPY NUMBER CLONING VEHICLE.

vector = vehicle - will discuss 3 or 4



FOREIGN DNA is spliced into the *pSC101* plasmid and introduced with the plasmid into the bacterium *Escherichia coli*. The plasmid is cleaved by the endonuclease *Eco RI* at a single site that does not interfere with the plasmid's genes for replication or for resistance to tetracycline (*top left*). The nucleotide sequence recognized by *Eco RI* is present also in other DNA, so that a foreign DNA ex- [illegible] to the [illegible] is cleaved about once in every 4,000 to 16,000 nucleotide pairs on a random basis (*top right*). Fragments of cleaved foreign DNA are annealed to the plasmid DNA by hydrogen bonding of the complementary base pairs, and the new composite molecules are sealed by DNA ligase. The DNA chimeras, each consisting of the entire plasmid and a foreign DNA fragment, are introduced into *E. coli* by transformation, and the foreign DNA is replicated by virtue of the replication functions of the plasmid

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

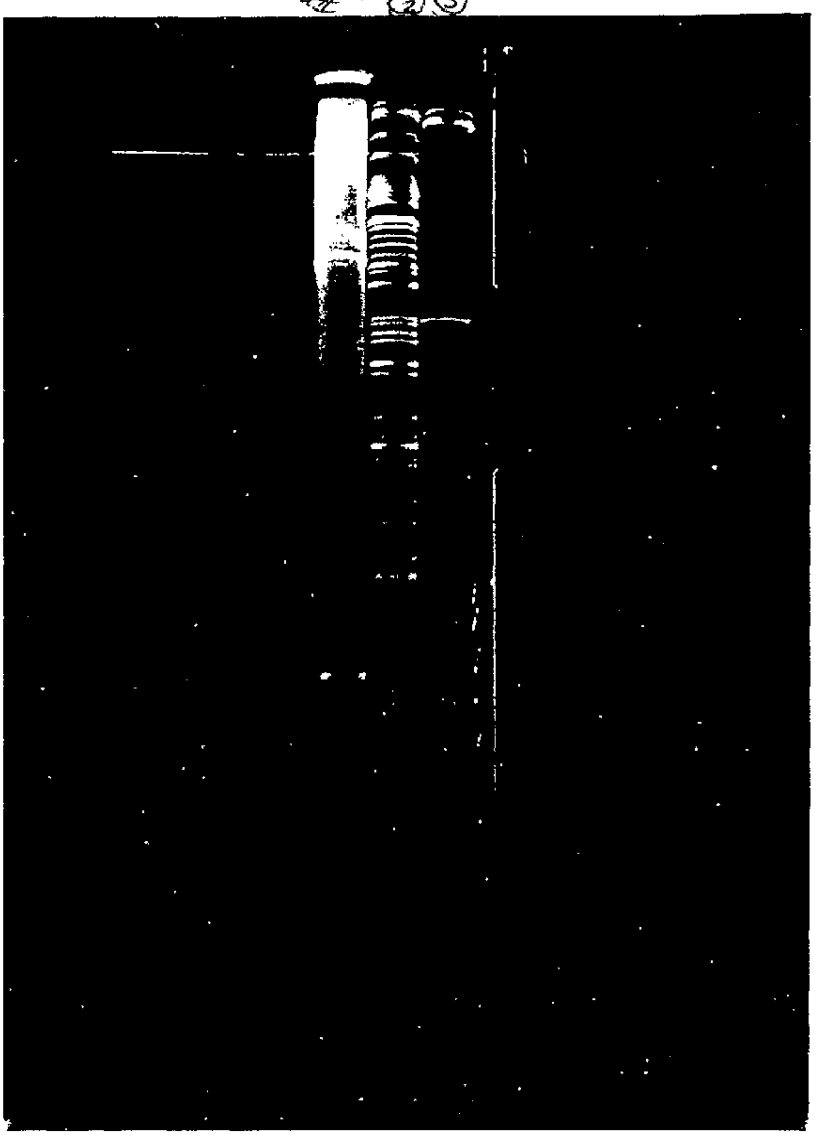
λ genome = 40,000 bp

FIG 60- PAGE ANALYSIS OF THE RESULTS OF CLONING FOLLOWING ONE FRAGMENT FROM A RESTRICTION DIGEST OF CHROMOSOMAL TO BACTERIOPHAGE TO PLASMID DNA.

① E. coli Hind III ② ③

② = λ Hind III

③ plasmid = 4000 bp Hind III



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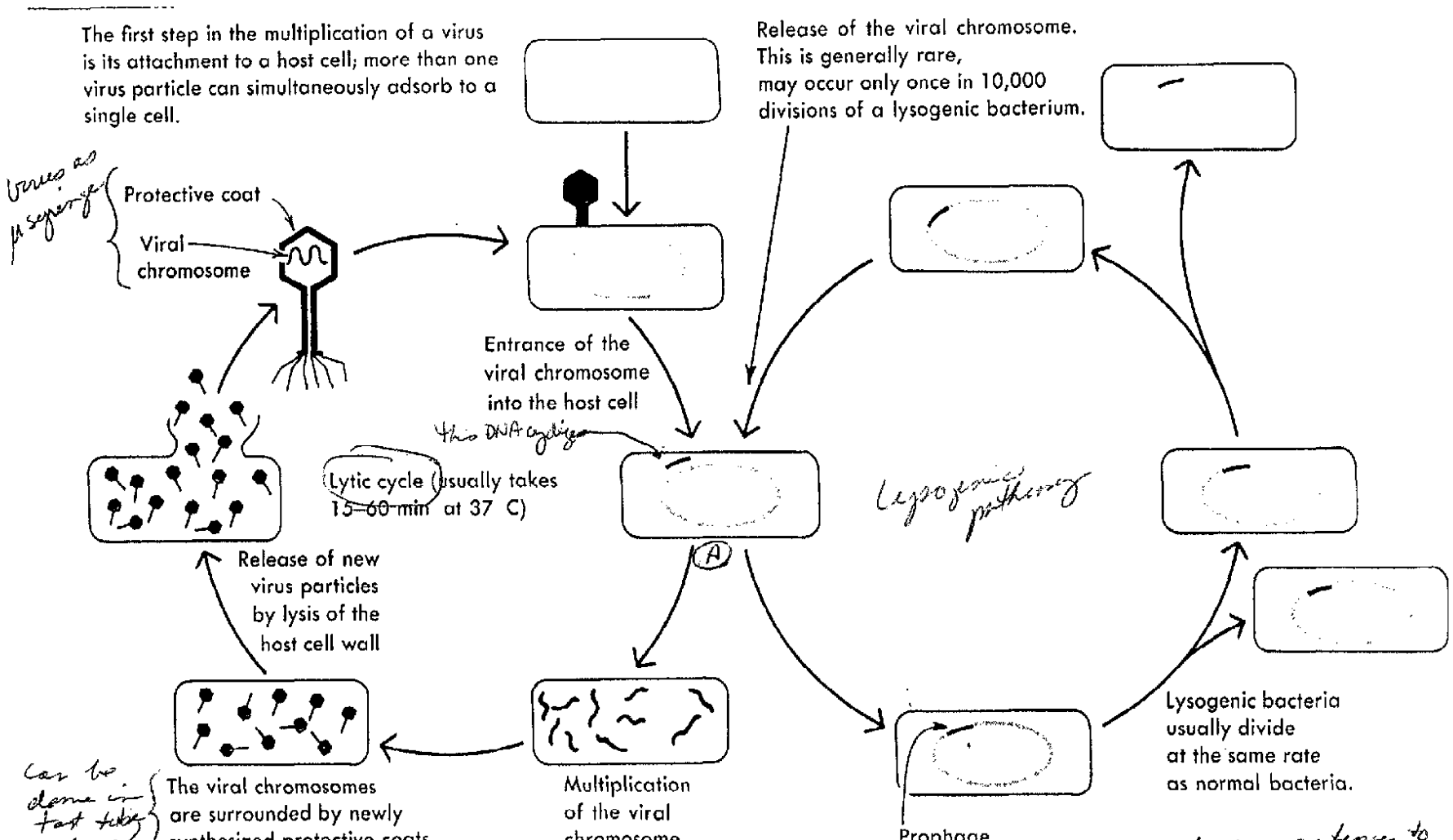
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Bacteriophage λ

FIG 61- BACTERIOPHAGE (BACTERIAL VIRUS) LAMBDA HAS TWO POSSIBLE LIFE CYCLES AFTER IT INFECTS A CELL. ITS DNA COULD FORM A STABLE INHERITED ASSOCIATION WITH THE HOST GENOME (THE LYSOGENIC PATHWAY) OR IT COULD MAKE AN INFECTIOUS CENTER (THE LYTIC PATHWAY).



(A) Either viral functions harmful to host are turned off + continues to reproduce or DNA copies + ~~[illegible]~~ takes over cell mech

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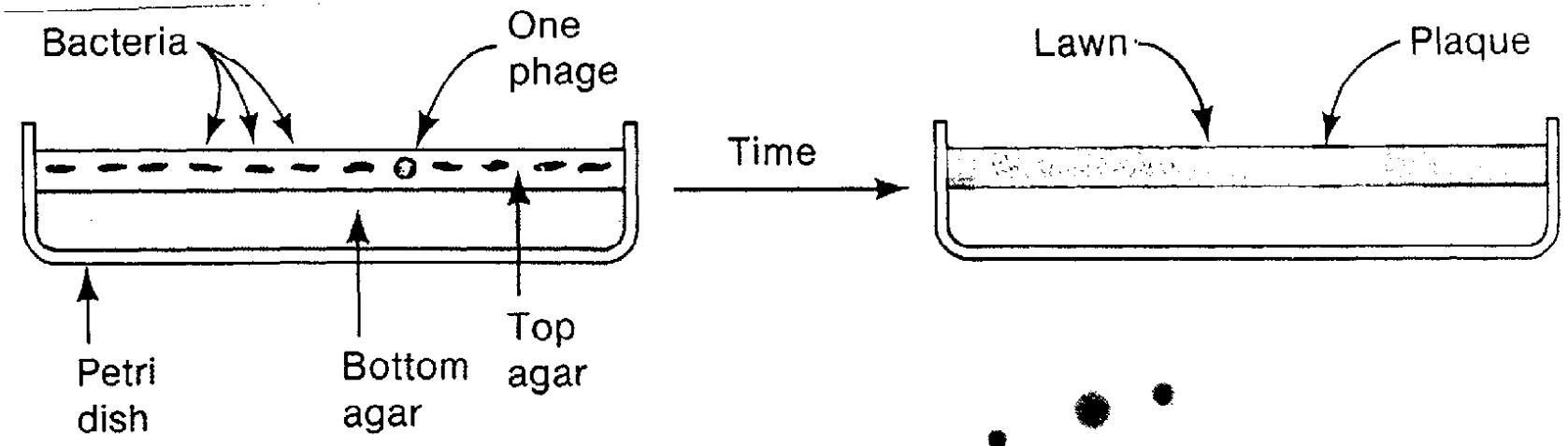
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Plaques here means clear zone of a bacterial lawn

FIG 62- BACTERIOPHAGE INFECTIOUS UNITS ARE IDENTIFIED BY A "PLAQUE" ASSAY.



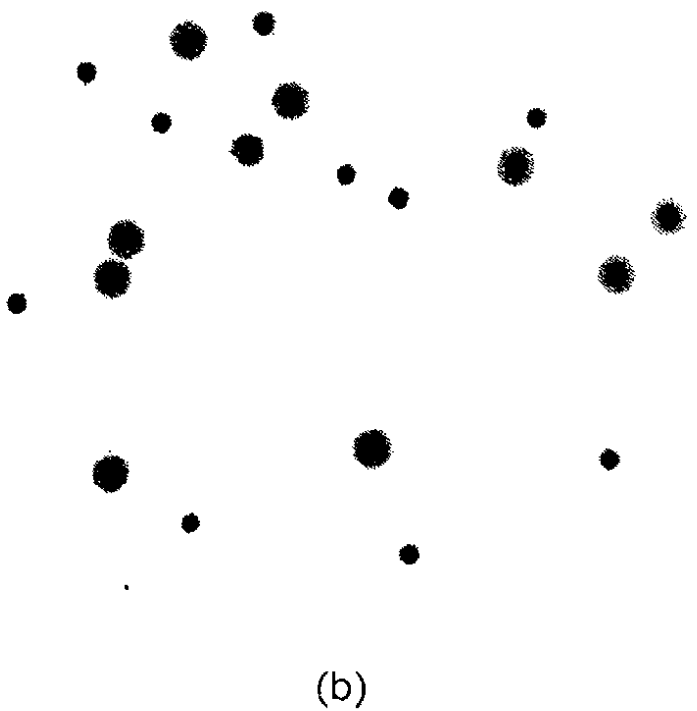


Figure 1-7 (a) Schematic drawing of plaque formation. Bacteria grow and form a translucent lawn. There are no bacteria in the vicinity of the plaque, which remains transparent. Neither phage nor bacteria are drawn to scale; both are much smaller. (b) Two types of plaques of phage T4: small, wild type; large, *rII* mutant. The illumination used for photography makes the plaques appear dark against a light background. [Courtesy of A. H. Doermann.]

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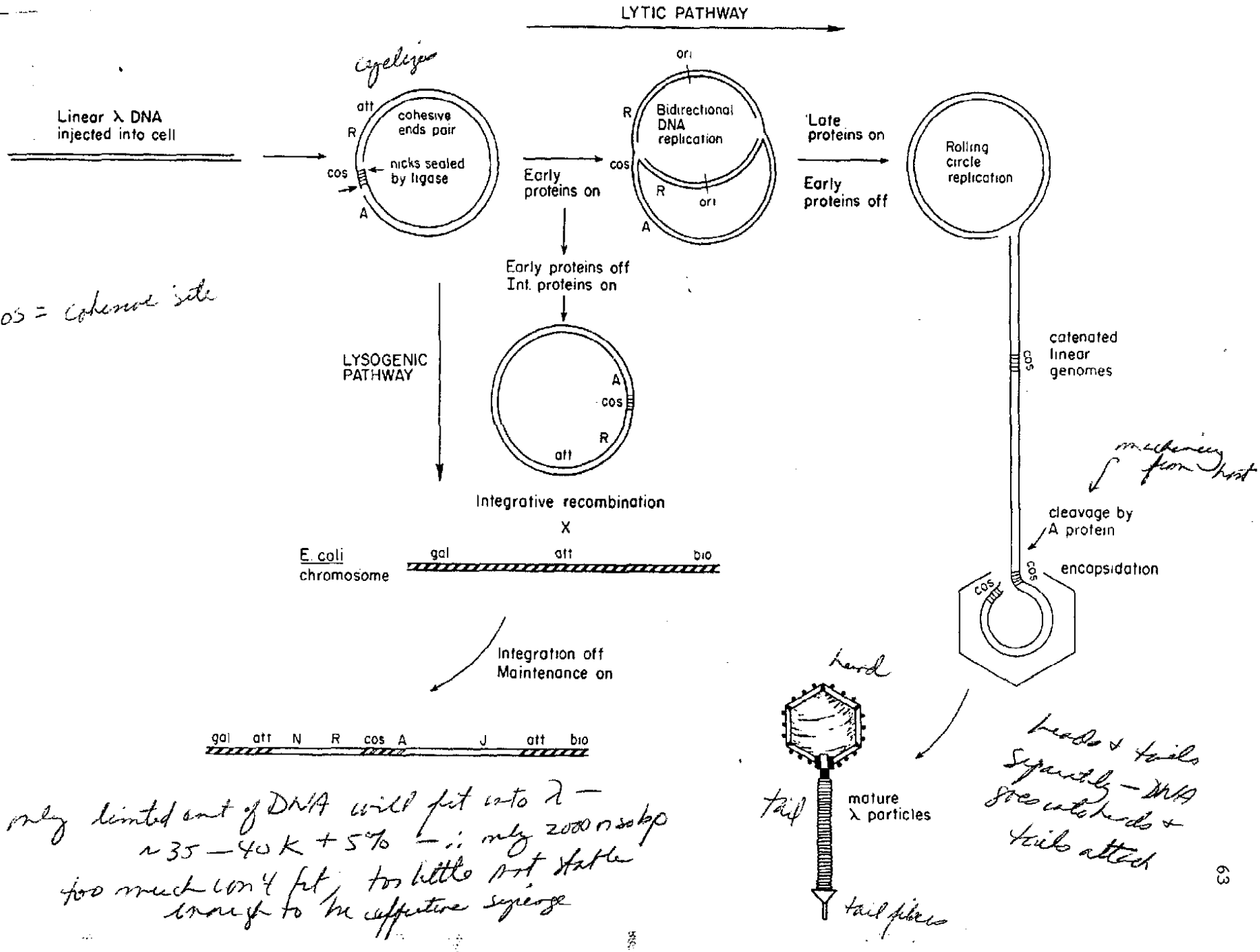
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 63- BACTERIOPHAGE LAMBDA PACKAGES ITS DNA INTO PHAGE PARTICLES BY A HEAD-FULL MECHANISM.



cos = cohesive site

only limited amt of DNA will fit into λ —
~35–40 K + 5% —∴ only 2000 nucleotides
too much can't fit, too little not stable
enough to be effective synergy

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63

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

regulatory + capsid are only genes required for lytic pathway. Lysogenic pathway genes can be eliminated for cloning purposes ≅ 15 kb. Need something more to make a stable particle, I think.

FIG 64- STRUCTURE OF THE BACTERIOPHAGE LAMBDA AND A LAMBDA CLONING VEHICLE

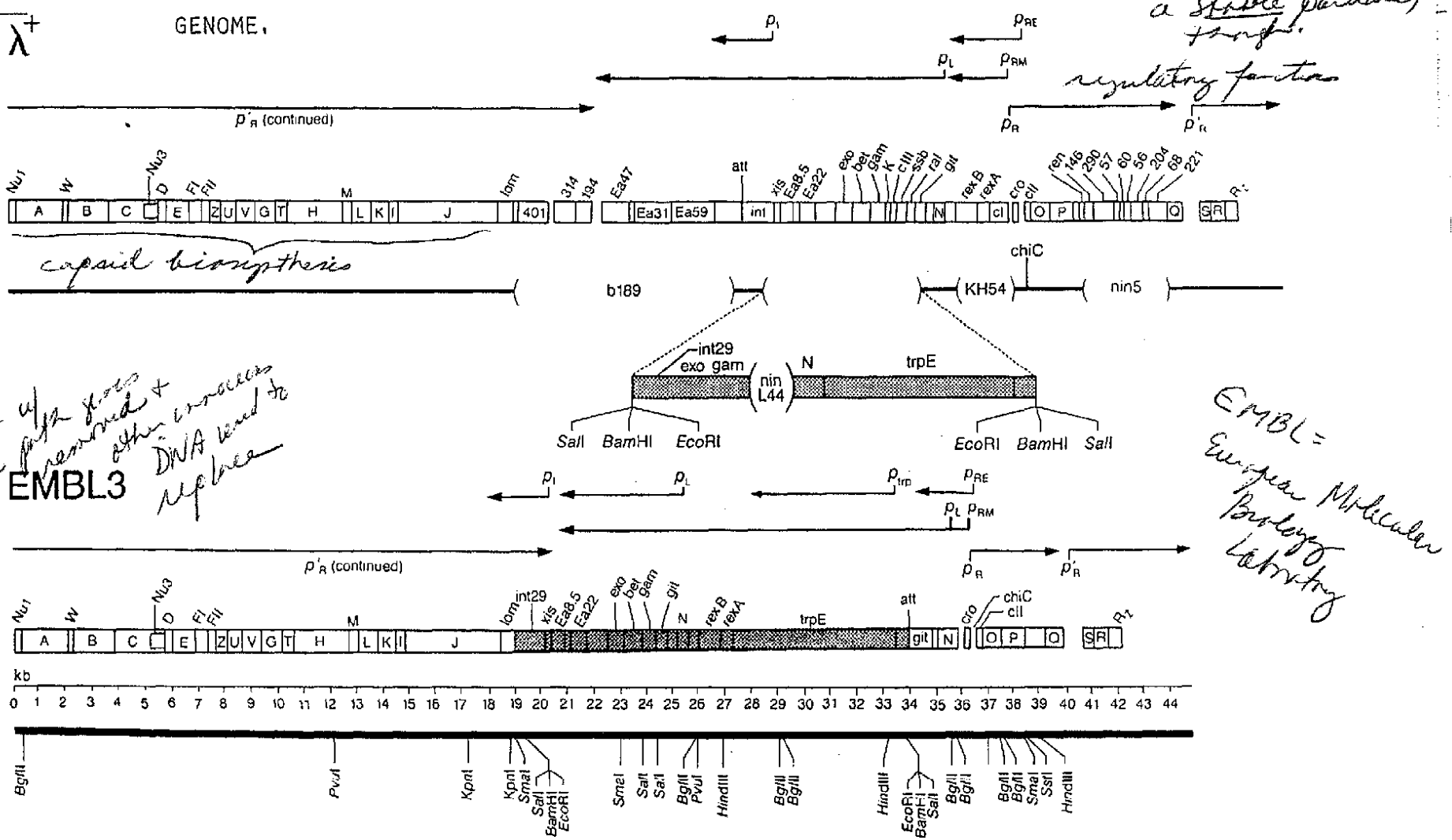

Figure 1.10.1 Wild-type lambda. Role of the major phage transcripts (arrows) and phage genes (boxes) in the lifecycle of bacteriophage λ is described in *UNITS 1.9* and *1.10*.

Figure 1.10.2 λ EMBL3. This phage vector is used for cloning large (10.4 to 20 kb) fragments. It contains a polylinker with cloning sites for *Bam*HI, *Sal* I, and *Eco*RI; in EMBL4, the polylinker is reversed. Recombinant phages containing DNA inserted into these sites become *cI*⁻, *gam*⁻, *red*⁻, and *int*⁻, and thus have the Spi⁻ phenotype. Although the phage is said to carry cI857, we believe that it does not carry a *cI* gene, and it has been drawn accordingly. The polylinker sequence is GGATCTGGGTCGACGGATCCGGGGAATTCCCAGATCC. EMBL4's full genotype is *λsbhlλ1° b189 < polylinker (SalI-EcoRI) int29 ninL44 cI857 trpE polylinker (EcoRI-SalI)> KH54 chiC srlλ4° nin5 srlλ5°* (Frischauff et al., 1983).

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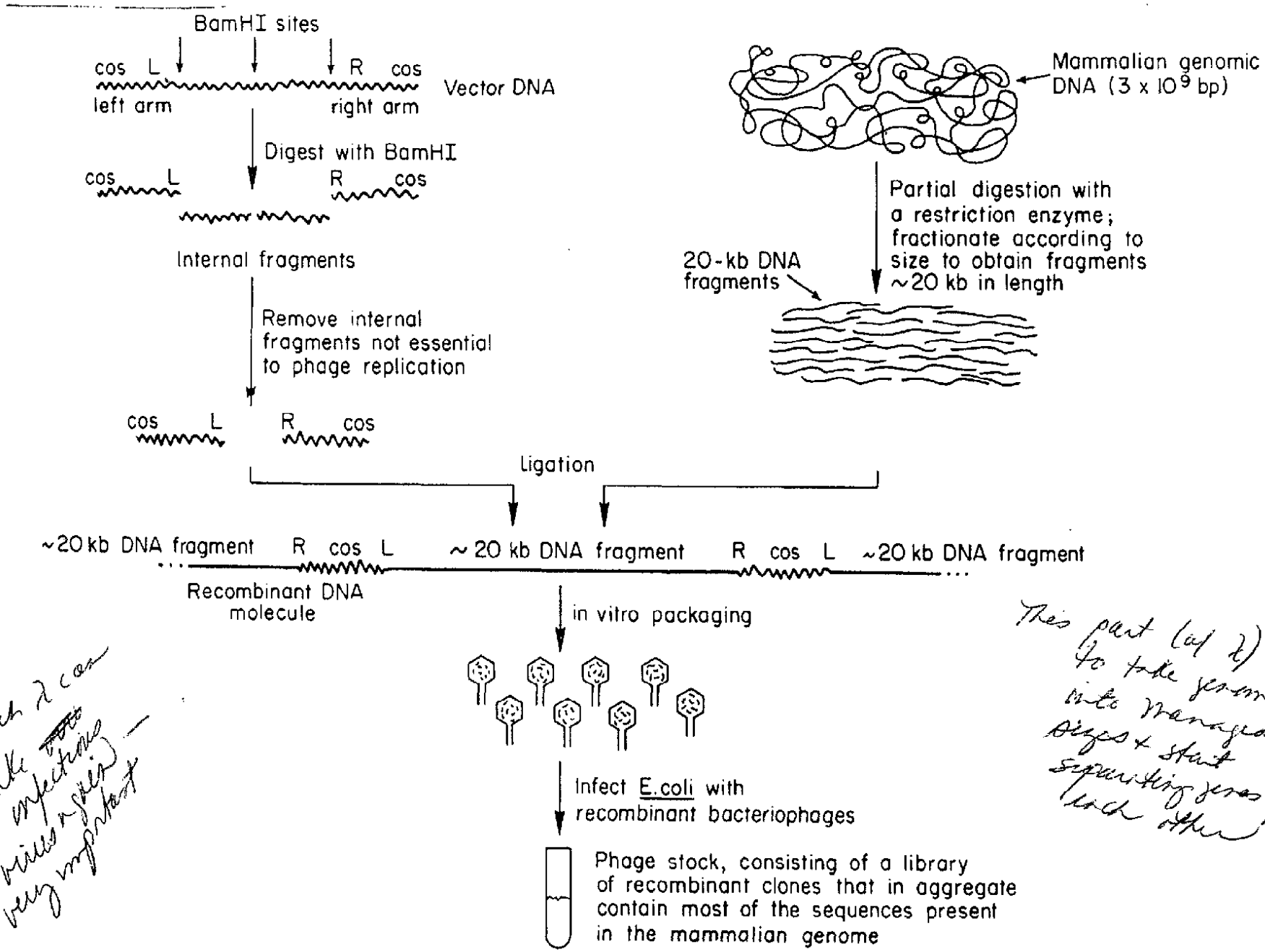
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 65- USING BACTERIOPHAGE LAMBDA AS A CLONING VECTOR.



λ good for particles 15-20 K bp
Good source of DNA

This part (w/ λ) is to take genome into manageable sizes + start separating genes from each other

each λ can make infectious virus in gain very important

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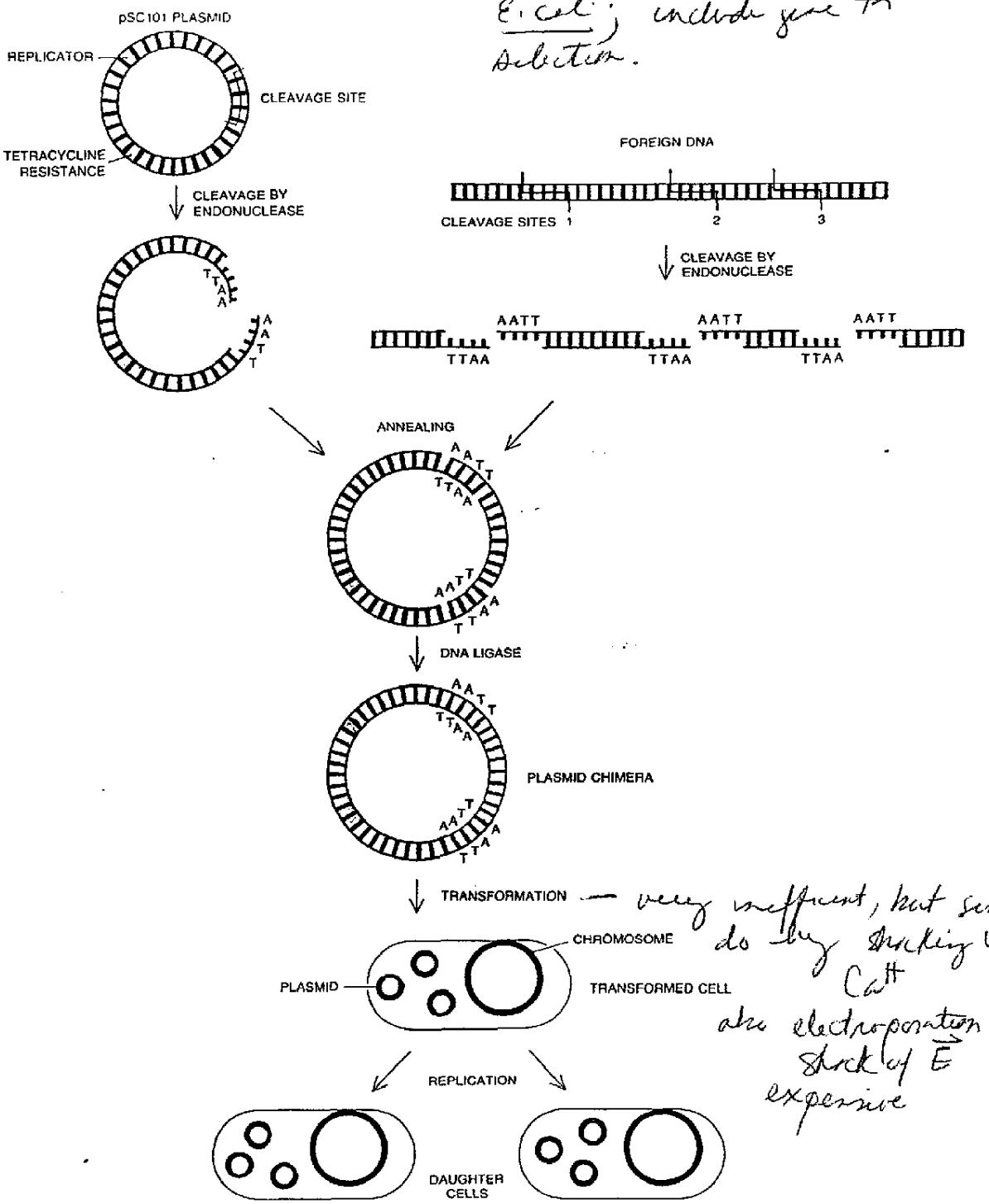
Plasmid another cloning vector. Come in variety of sizes. Usually small, simple ones found in multiple copy in all cell for recomb DNA 3000 – 10,000 bp.; origin of rep compatible w/ E. coli; include gene for selection.

FIG 66- USING A MULTICOPY PLASMID AS A CLONING VECTOR.



— very inefficient, but simple do by shocking w/ Ca^{++} also electroporation — shock of $\vec{E}$ expensive

FOREIGN DNA is spliced into the *pSC101* plasmid and introduced with the plasmid into the bacterium *Escherichia coli*. The plasmid is cleaved by the endonuclease *Eco RI* at a single site that does not interfere with the plasmid's genes for replication or for resistance to tetracycline (*top left*). The nucleotide sequence recognized by *Eco RI* is present also in other DNA, so that a foreign DNA exposed to the endonuclease is cleaved about once in every 4,000 to 16,000 nucleotide pairs on a random basis (*top right*). Fragments of cleaved foreign DNA are annealed to the plasmid DNA by hydrogen bonding of the complementary base pairs, and the new composite molecules are sealed by DNA ligase. The DNA chimeras, each consisting of the entire plasmid and a foreign DNA fragment, are introduced into *E. coli* by transformation, and the foreign DNA is replicated by virtue of the replication functions of the plasmid.

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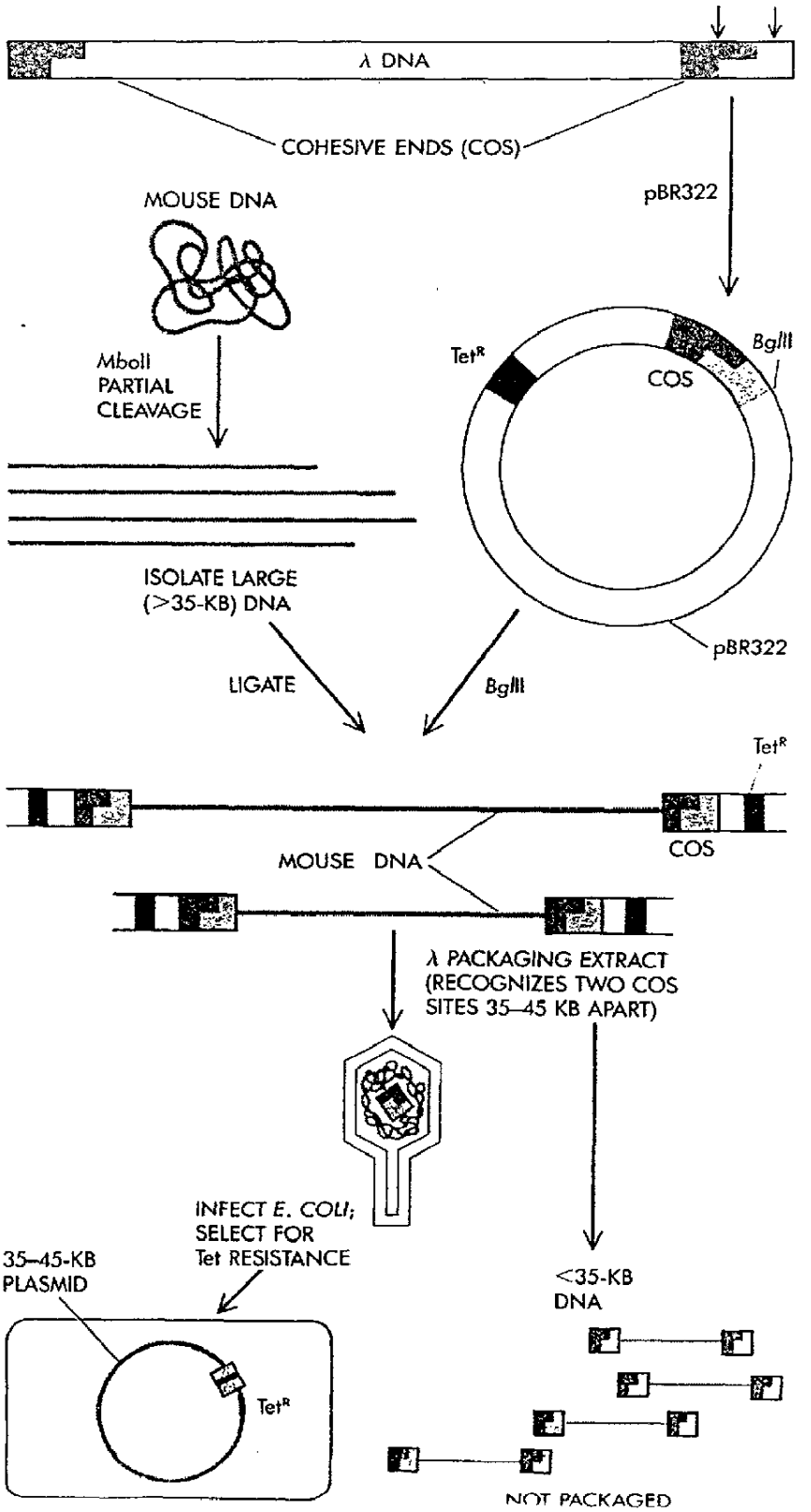
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Skipped

FIG 67- COSMIDS ARE PLASMIDS WHICH CONTAIN "COS" SITES FROM LAMBDA. COSMID CLONING PERMITS THE CLONING OF VERY LARGE DNA FRAGMENTS AND TAKES ADVANTAGE OF THE HIGH EFFICIENCY OF INTRODUCTION INTO THE HOST CHARACTERISTIC OF THE LAMBDA PACKAGING SYSTEM.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

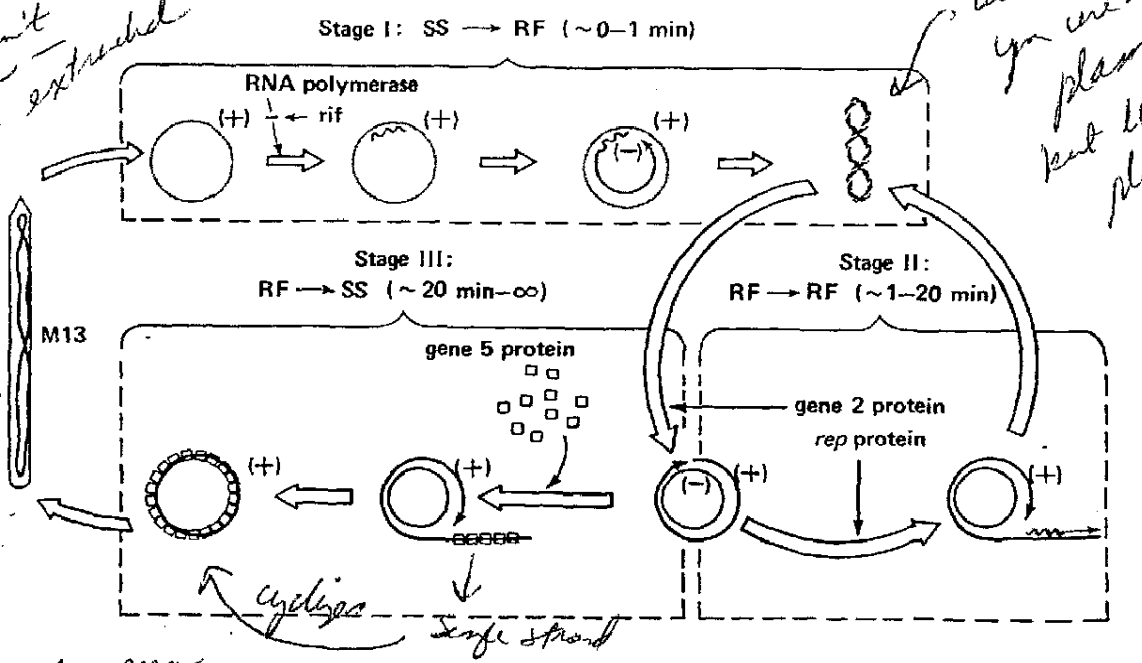
M13 another virus

FIG 68A- BACTERIOPHAGE M13'S LIFE CYCLE ALLOWS ONE TO ISOLATE BOTH DOUBLE STRANDED AND SINGLE STRANDED VERSIONS OF CLONED GENES.

6–10 K bp

virus doesn't kill cell here — particles are extruded out.

only one of 2 strands present here



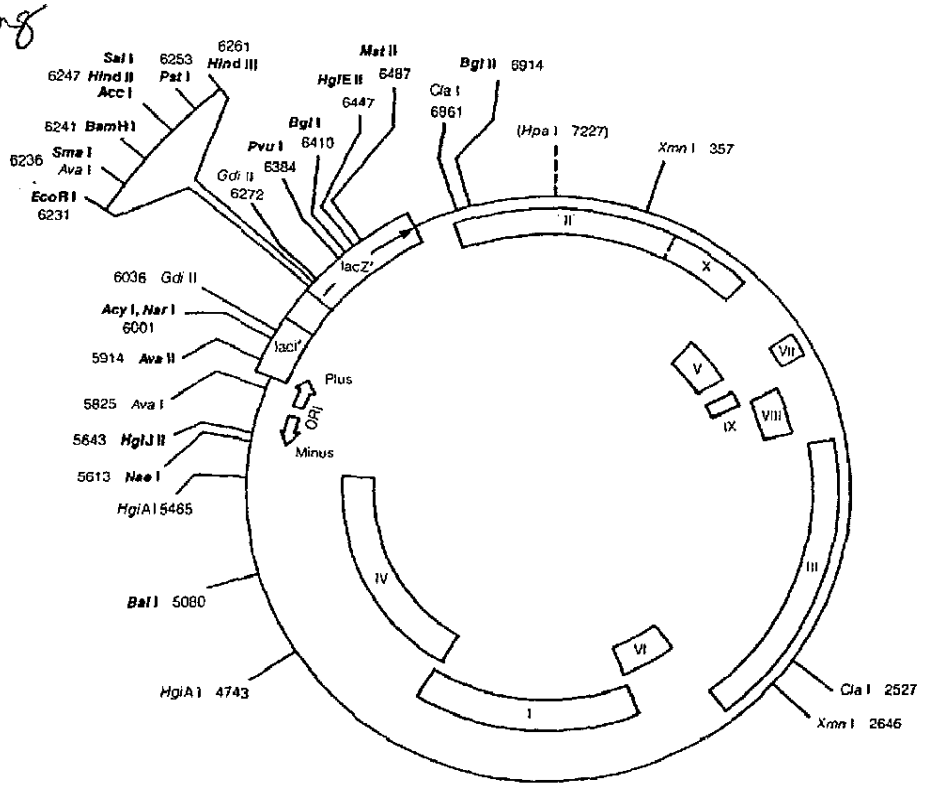
cyclizes

single strand

use this like you use a plasmid, but look for plaques

plaque may be seen if cells are dead or slowly growing

FIG 68B- BACTIOPHAGE M13 CLONING VECTOR.



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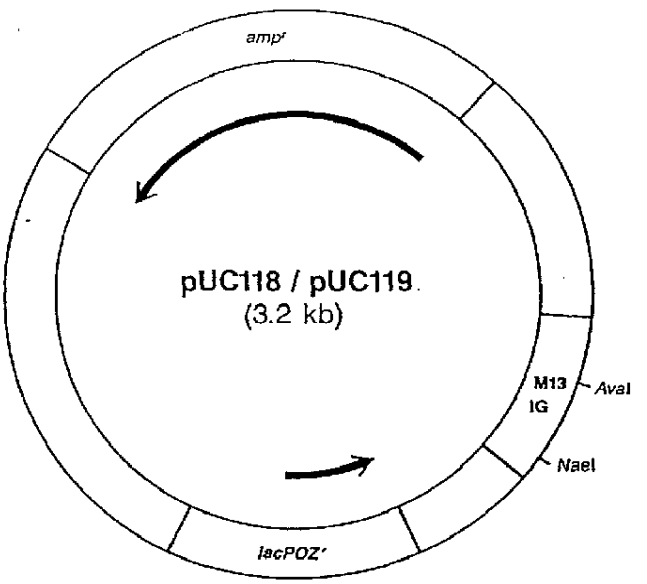
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001



Polycloning Sites

pUC118

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	7	8
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala	Ser	Leu	Ala	Leu	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA	AGC	TTG	GCA	CTG	GCC

EcoRI *SacI* *KpnI* *SmaI* *XmaI* *BamHI* *XbaI* *SalI* *AccI* *HincII* *PstI* *SphI* *HindIII*

pUC119

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	5	6	7	8
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser	Ser	Asn	Ser	Leu	Ala
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC	TCG	AAT	TCA	CTG	GCC

HindIII *SphI* *PstI* *SalI* *AccI* *HincII* *XbaI* *BamHI* *SmaI* *XmaI* *KpnI* *SacI* *EcoRI*

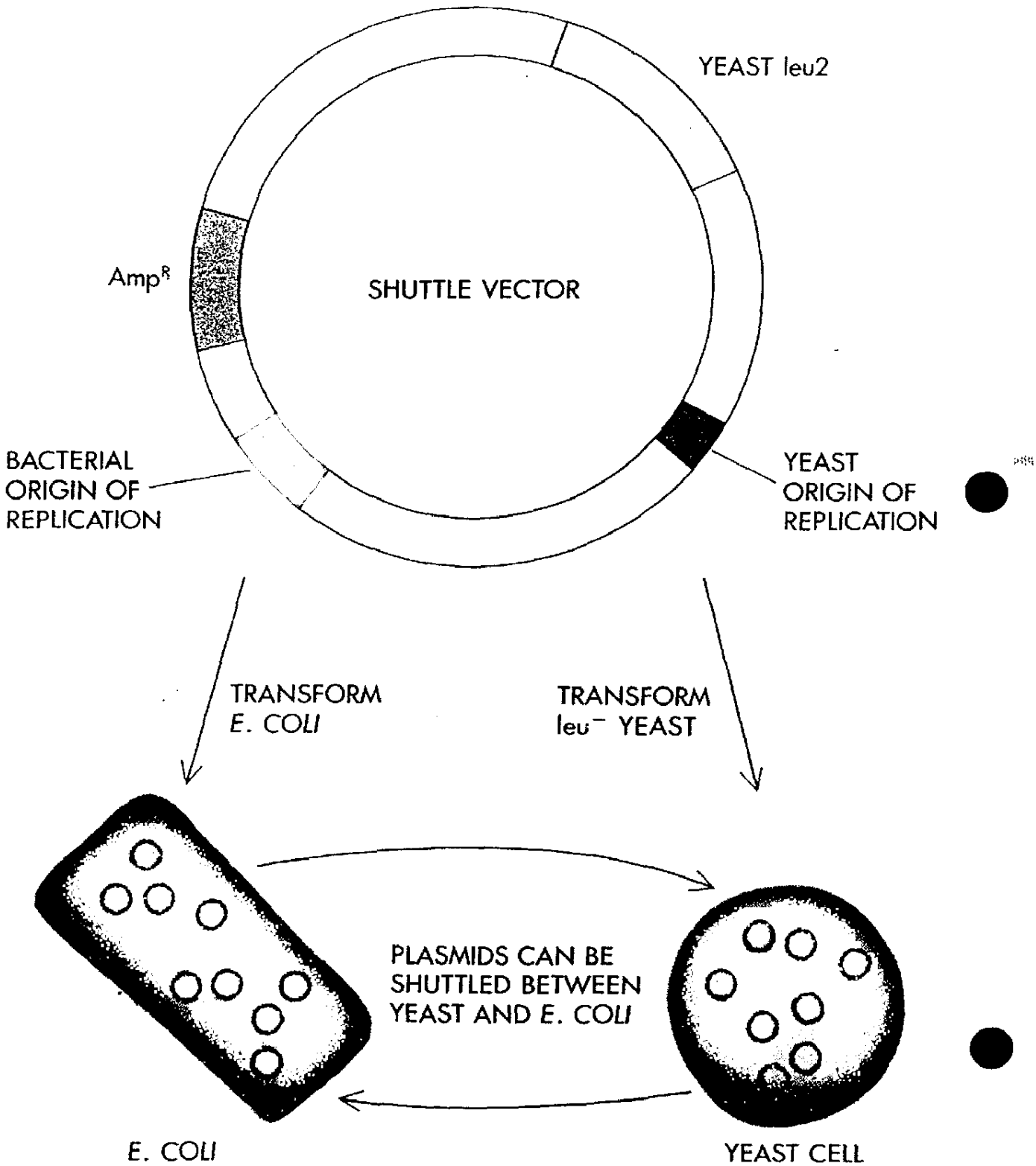
Fig 68a

Phagemid vectors pUC118 and pUC119. The positions of the unique sites in the 476-bp intergenic region are shown. Restriction sites in the remainder of the vector are the same as in pUC18 and pUC19.

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

= shuttle vctr

FIG 69- DUAL HOST VECTOR HAS ORIGINS OF REPLICATION FOR E. COLI AND YEAST AND SELECTABLE MARKERS FOR BOTH HOSTS.



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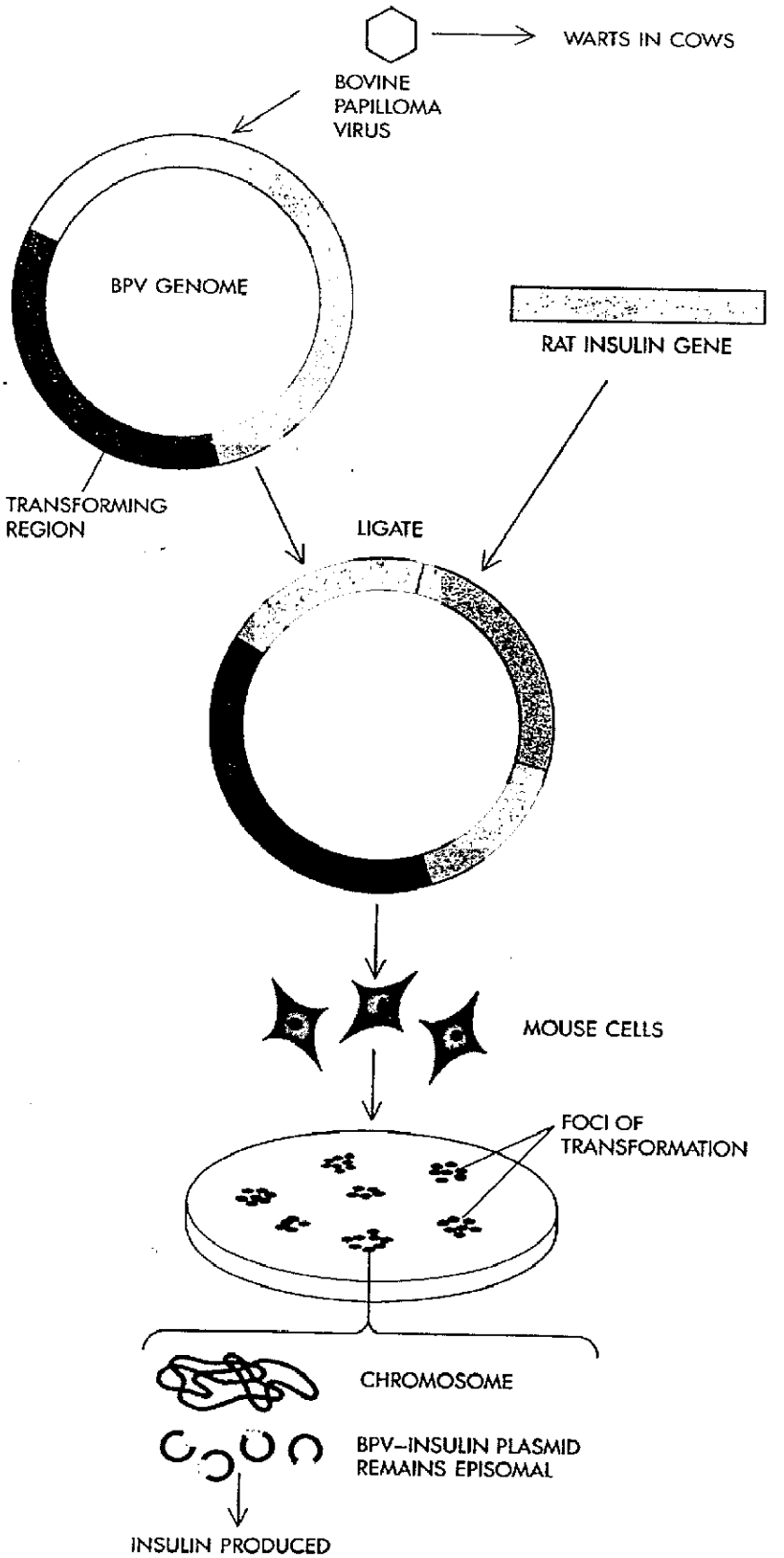
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

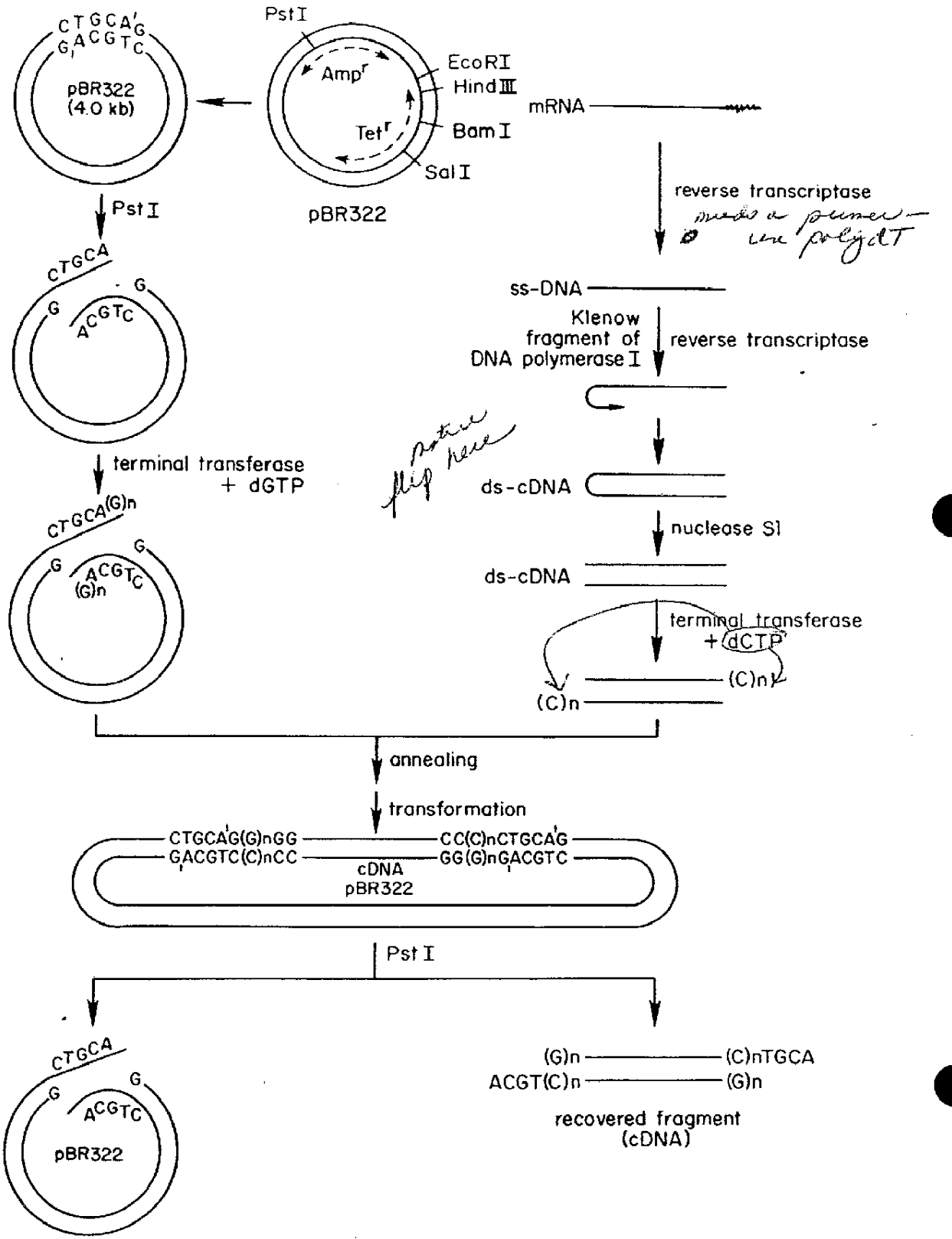
FIG 70- MAMMALIAN CELLS CAN RECEIVE FOREIGN DNA USING VECTORS SUCH AS THOSE DERIVED FROM BOVINE PAPILLOMA VIRUS.



Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

3' end of most eukaryotic messages is poly A.
∴ use poly T as primer to go backwards

FIG 71- RNA CAN BE USED AS A SOURCE OF DNA FOR CLONING THROUGH THE SYNTHESIS OF cDNA.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Purifying RNA for cDNA synthesis
① Choose right cells
② Immunoprecip of polyribosomes
③ Oligo dT columns
④ Size fractionation
⑤ In vitro translation assay
⑥ Negative hybridization – based on same concept as oligo dT columns See p. 73
⑦ Selective primers

MAKING THE CORRECT cDNA

(1) CHOOSE RIGHT CELLS AND GROWTH CONDITIONS FOR RNA SOURCE.

(2) PURIFY RNA USING: OLIGO dT COLUMNS
SIZE FRACTIONATION
NEGATIVE HYBRIDIZATION
IMMUNOPRECIPITATION OF POLYRIBOSOMES

(3) ASSAY RNA PURITY THROUGH TRANSLATION ASSAYS.

(4) SYNTHESIZE SPECIFIC cDNA USING A DEFINED OLIGO AS A PRIMER.

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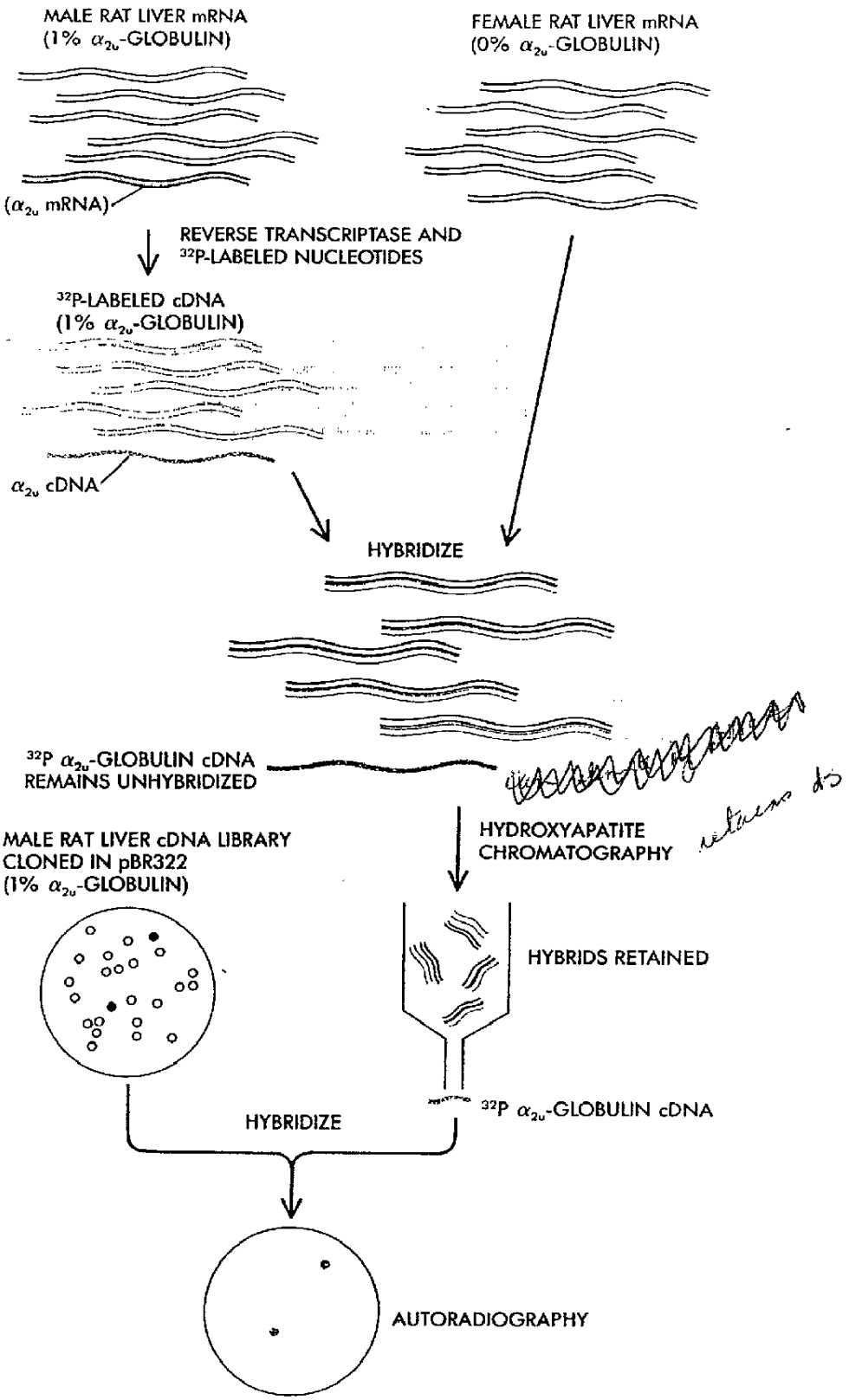
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 73 - PURIFYING SPECIFIC cDNA BY NEGATIVE HYBRIDIZATION.



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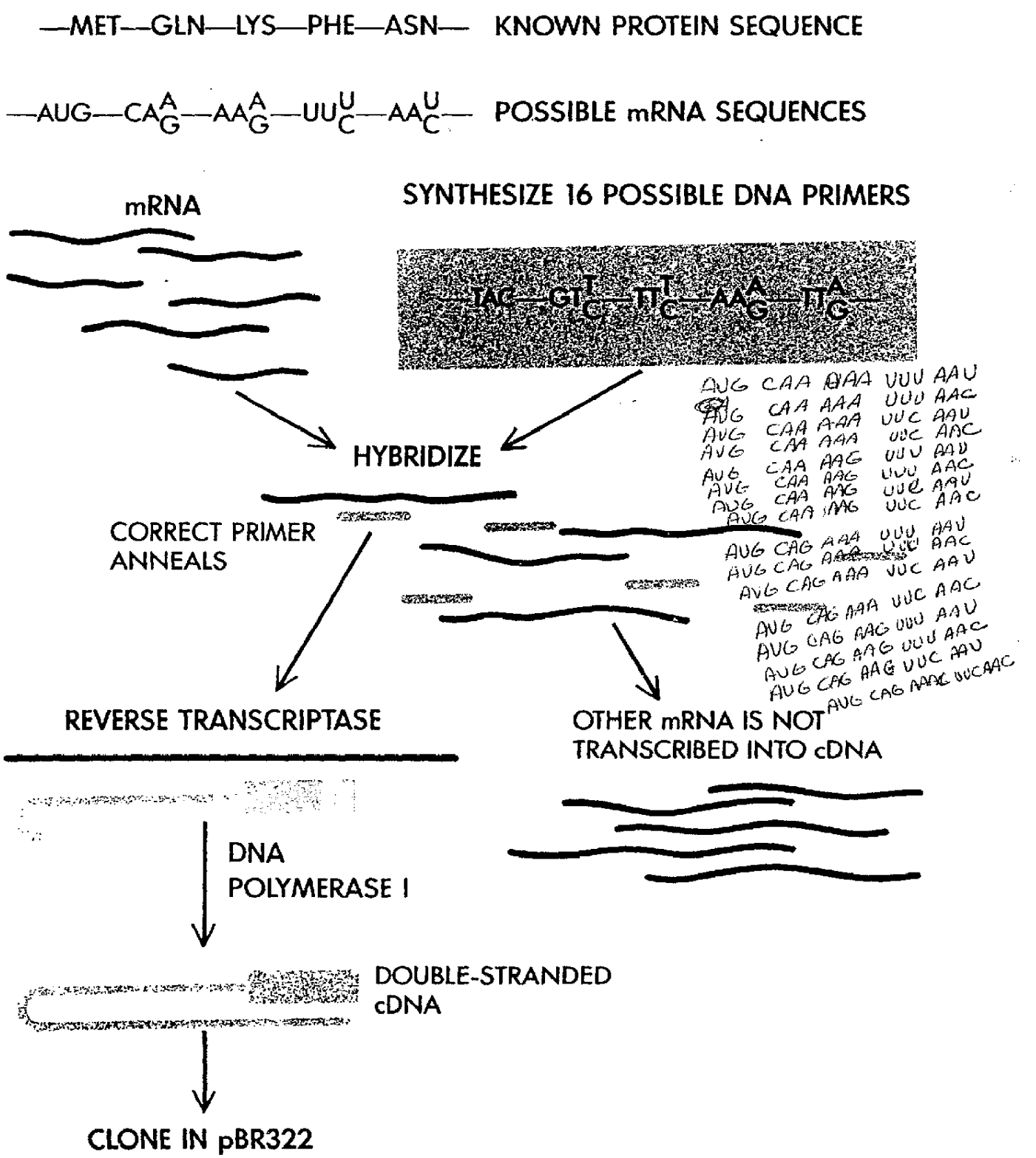
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 74 - SYNTHESIZING SPECIFIC cDNA USING DEFINED SEQUENCE PRIMER.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

For synthetic oligo practical limit ≈ 100 – 150 bp.

FIG 75A- CHEMICAL TECHNIQUES CAN BE USED TO SYNTHESIZE DEFINED DNA SEQUENCES.

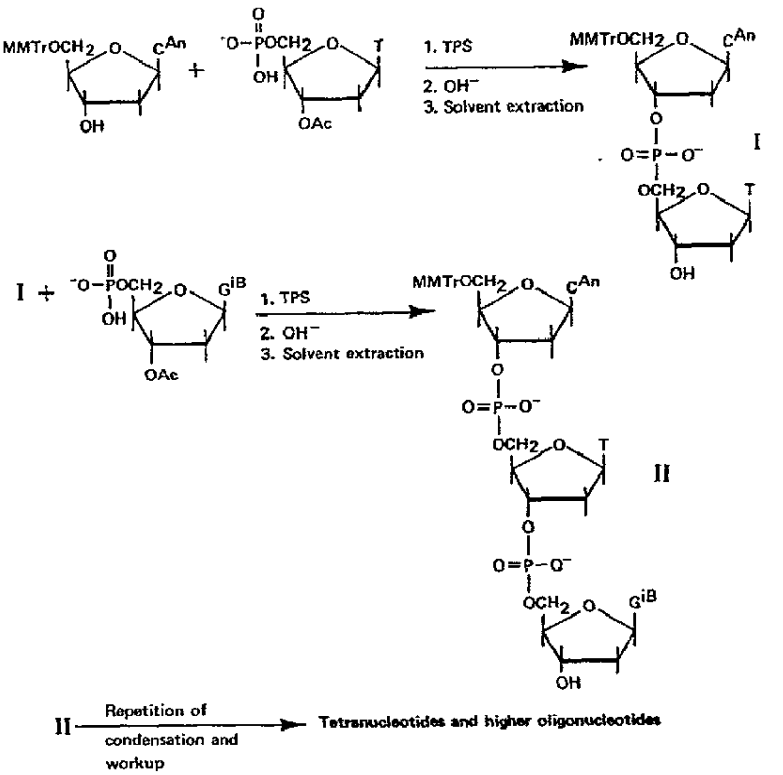
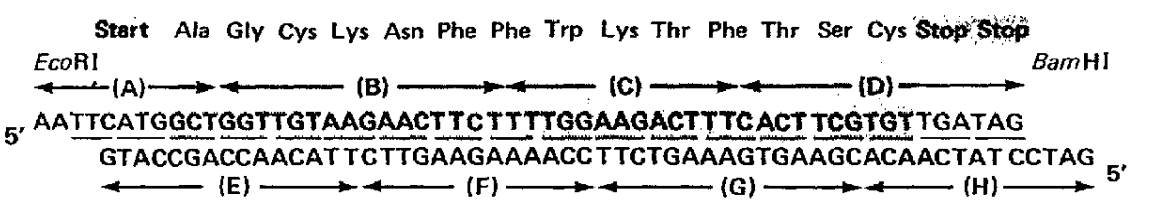


FIG 75B- SYNTHETIC DNA SEQUENCES CAN BE LIGATED TOGETHER TO MAKE A GENE.



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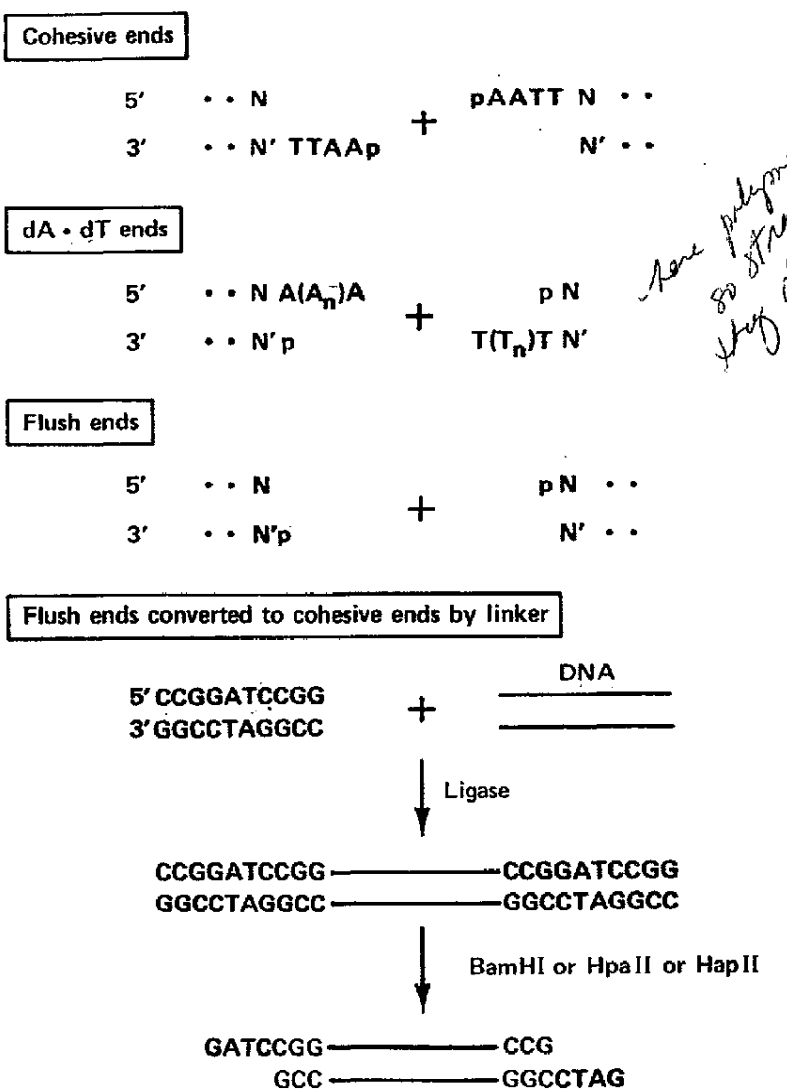
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 76- COHESIVE, BLUNT AND "ARTIFICIAL" COHESIVE ENDS CAN BE USED IN CLONING.



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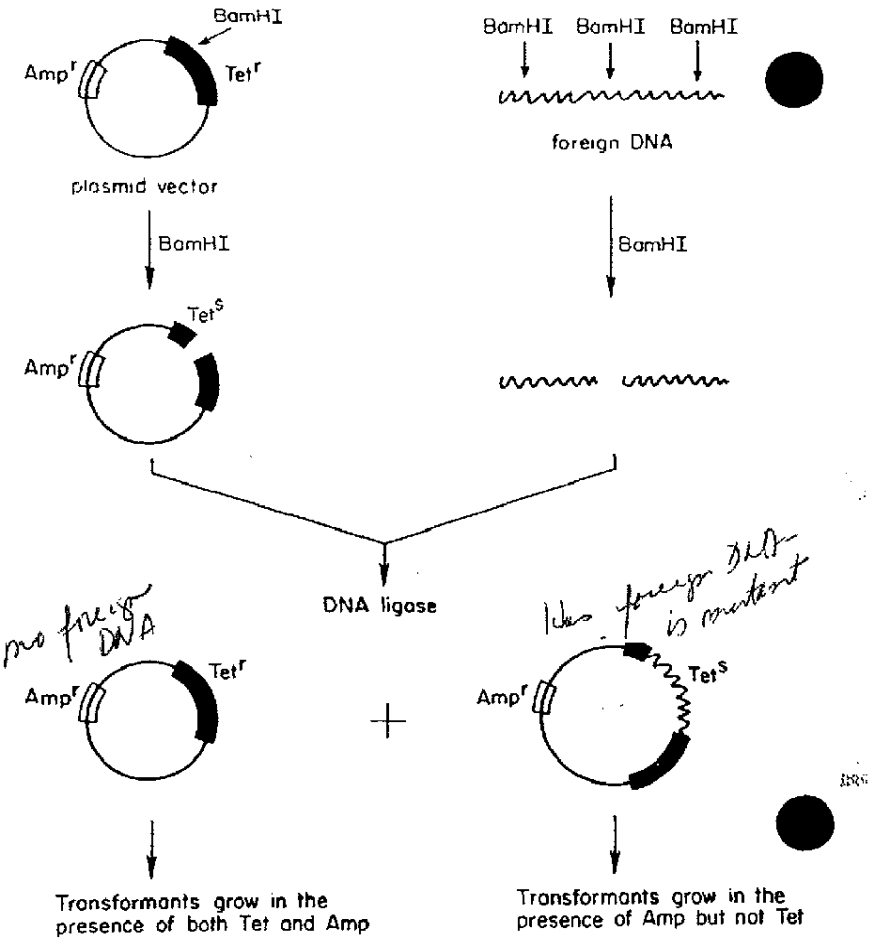
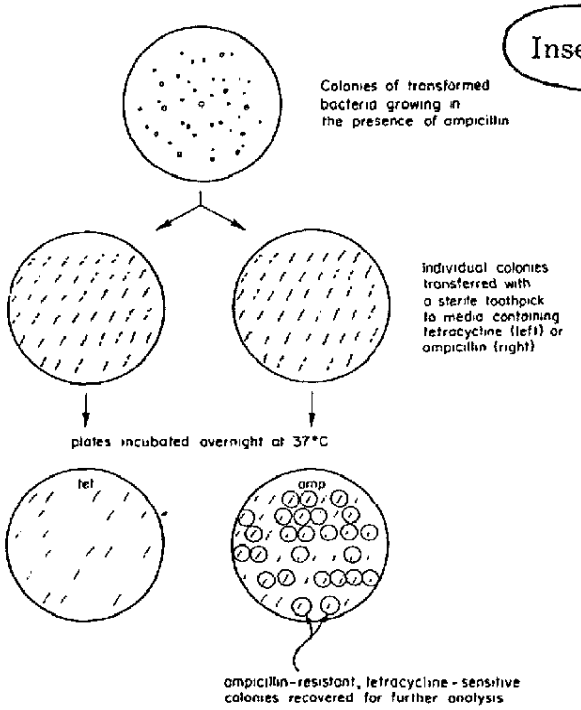
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 77- SUCCESSFUL CLONING OF ANY DNA CAN BE DETECTED BY LOSS OF A FUNCTION ENCODED BY THE VECTOR.





Screening for insertions of foreign DNA by inactivation of plasmid-borne, antibiotic-resistance genes.

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

5' PO4 required for ligase function

FIG 78- USING PHOSPHATASE TO PREVENT SELF LIGATION. LIGASE ONLY WORKS ON DNA MOLECULES WITH 5' PO_4 GROUPS. BY USING PHOSPHATASE TO REMOVE THE 5' PO_4 GROUPS FROM THE VECTOR, SELF-CLOSURE OF THE VECTOR CAN BE PREVENTED.

∴ only get insert!

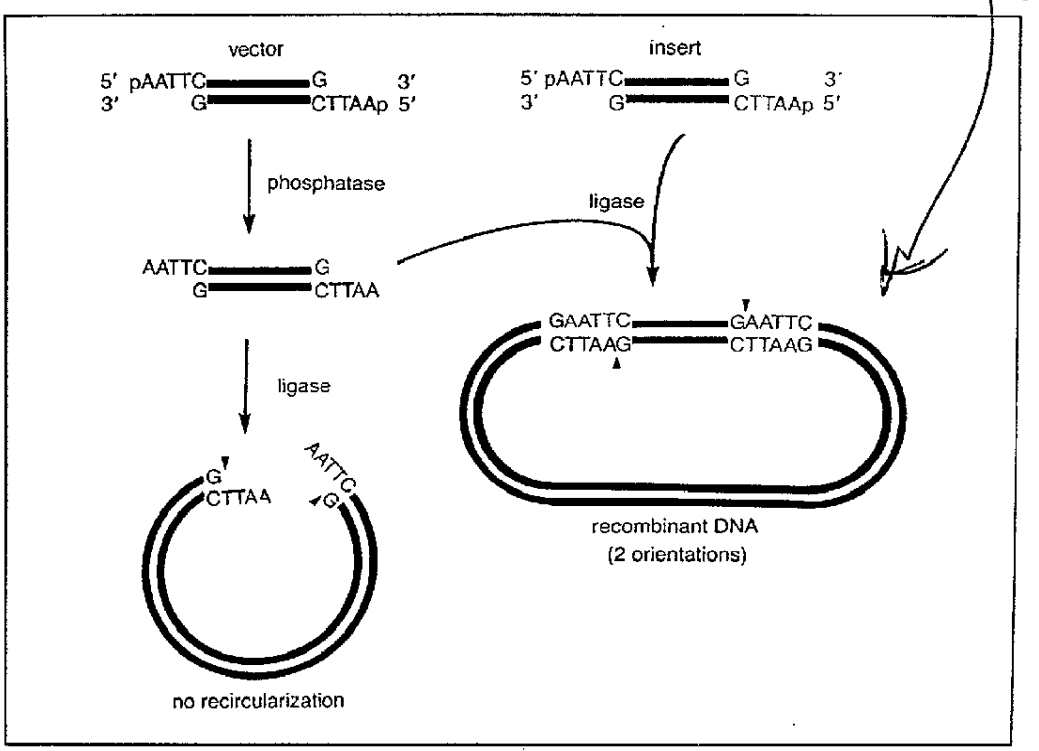

Figure 3.16.1 Treatment of cleaved vector with CIP in order to reduce background.

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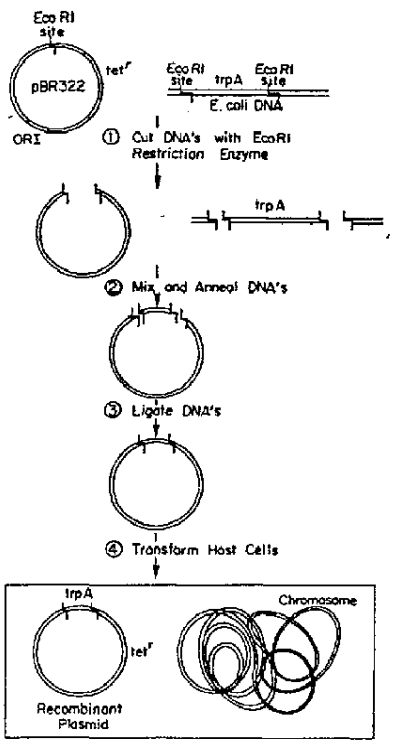
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Skip

FIG 79- SOME RECOMBINANT DNA MOLECULES CAN BE DIRECTLY SELECTED IN THE APPROPRIATE HOST IF THE CLONED DNA PROVIDES AN EXPRESSED FUNCTION LACKING IN THE HOST.



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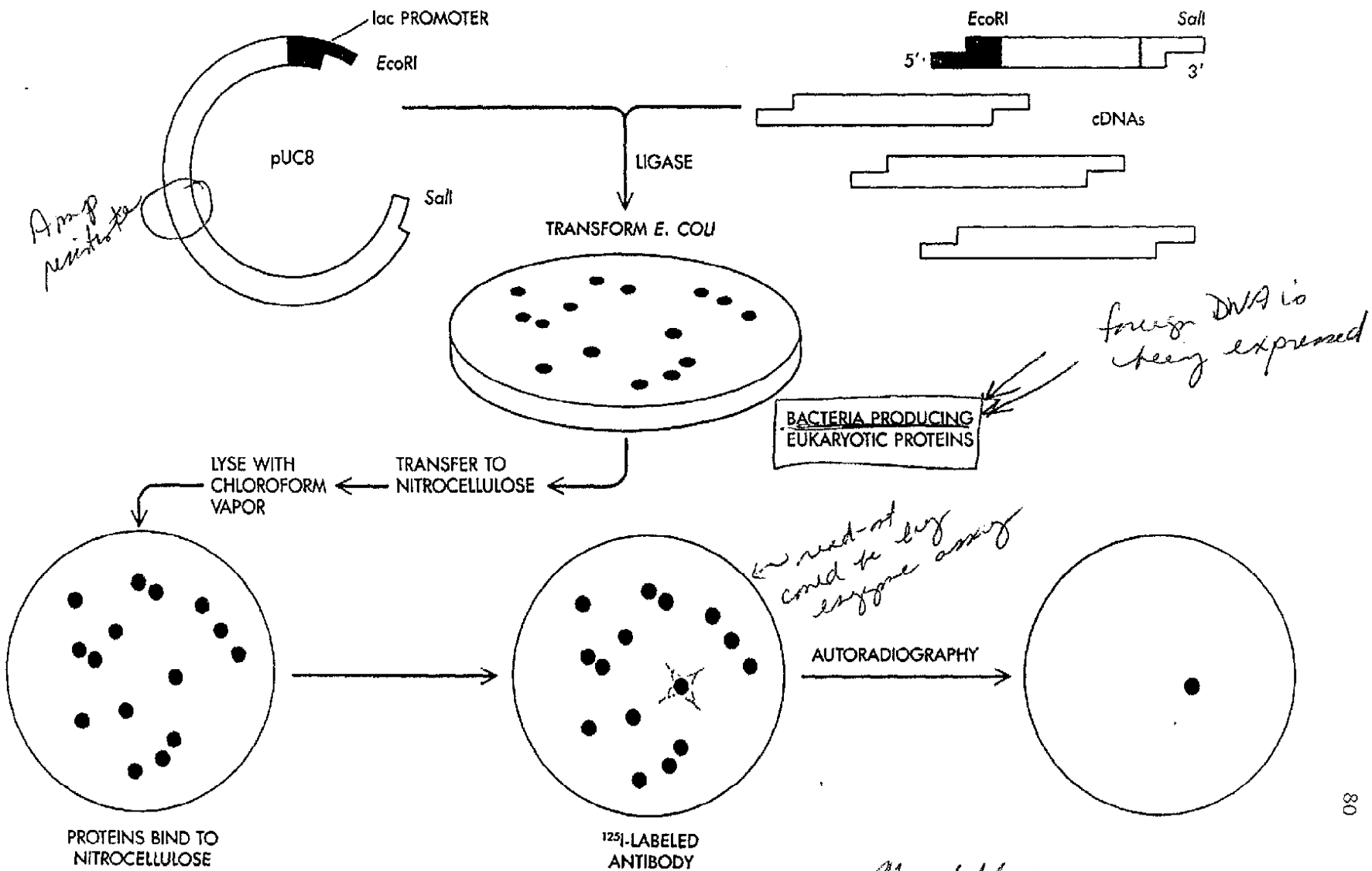
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 80- IF THE CLONED GENE PRODUCES AN ANTIGENIC PRODUCT, COLONIES CARRYING THE RECOMBINANT MOLECULE CAN BE DETECTED IMMUNOLOGICALLY.



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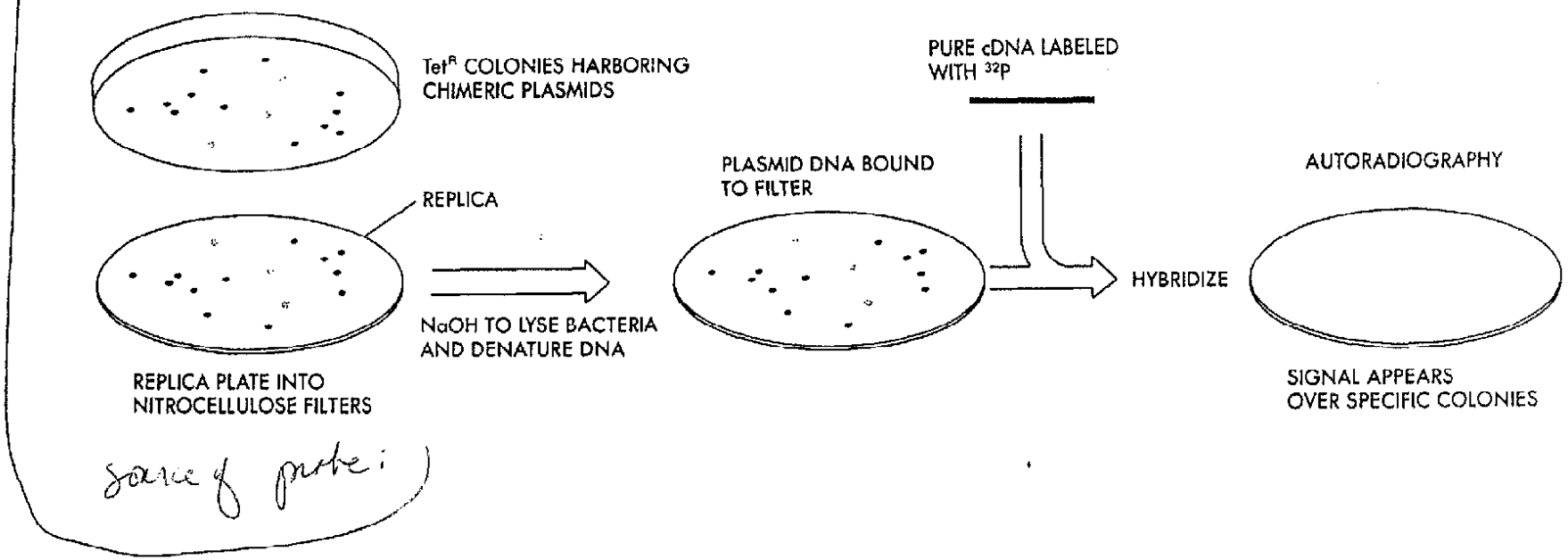
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

chimeric = recombinant

can do some type of plaques

FIG 81- SPECIFIC CLONED SEQUENCES CAN BE DETECTED BY THE FILTER HYBRIDIZATION TECHNIQUE. THE SOURCE OF THE RADIOACTIVE "PROBE" COULD INCLUDE:

(1) SMALL SECTION OF DNA (FROM PREVIOUS CLONE)
(2) RNA (OR cDNA)
(3) SYNTHETIC OLIGONUCLEOTIDE (DEDUCED FROM PROTEIN SEQUENCE) - most powerful
make the probe redundant to deal w/ degeneracy
Bull used synthetic oligos most of the time
- mentioned current work w/ sequence!



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

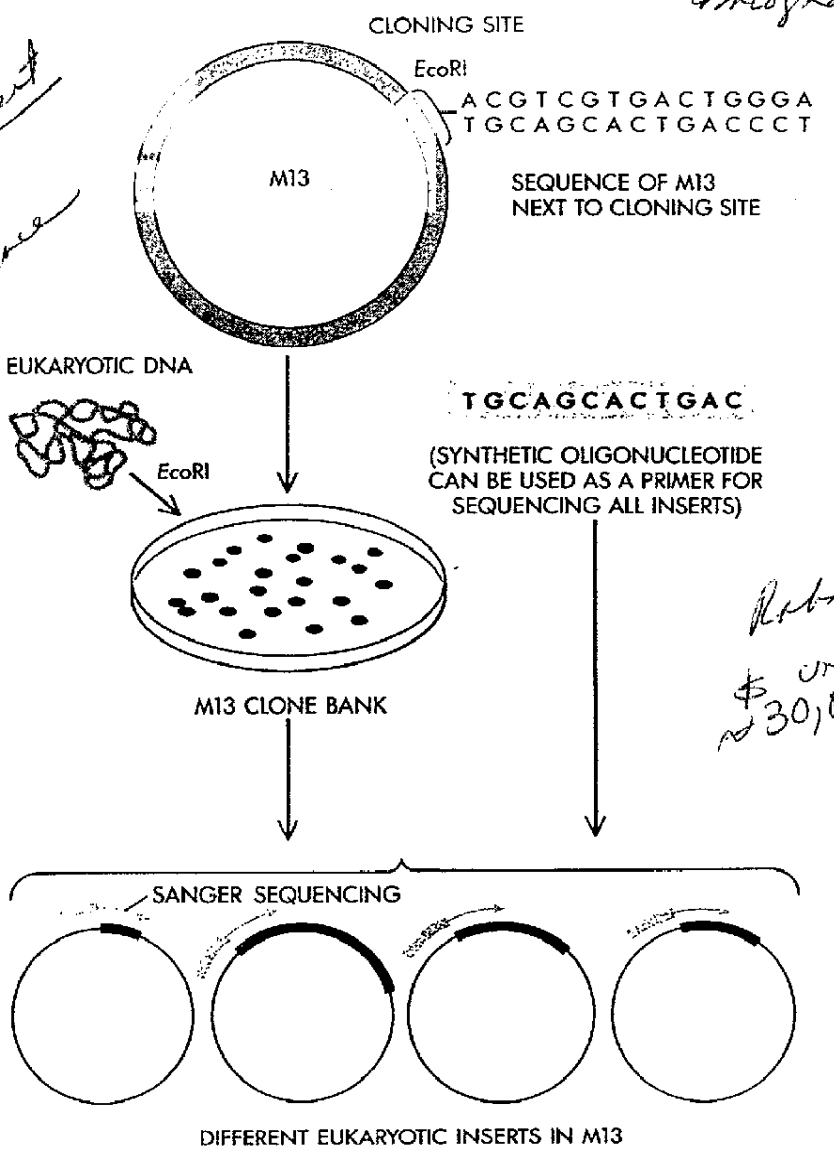
Have to sequence the clone even if ~~sequenced~~ synthesized the clone — otherwise may have protective groups that are alkylated — cause they look like they smoked or consumed NAs in bologna ...

FIG 82- THE PRODUCTS OF A CLONING EXPERIMENT CAN BE SCREENED BY DIRECT DNA SEQUENCE ANALYSIS.

cost of sequencing ~$1/base

sequence only insert not the vector

5μg DNA to sequence



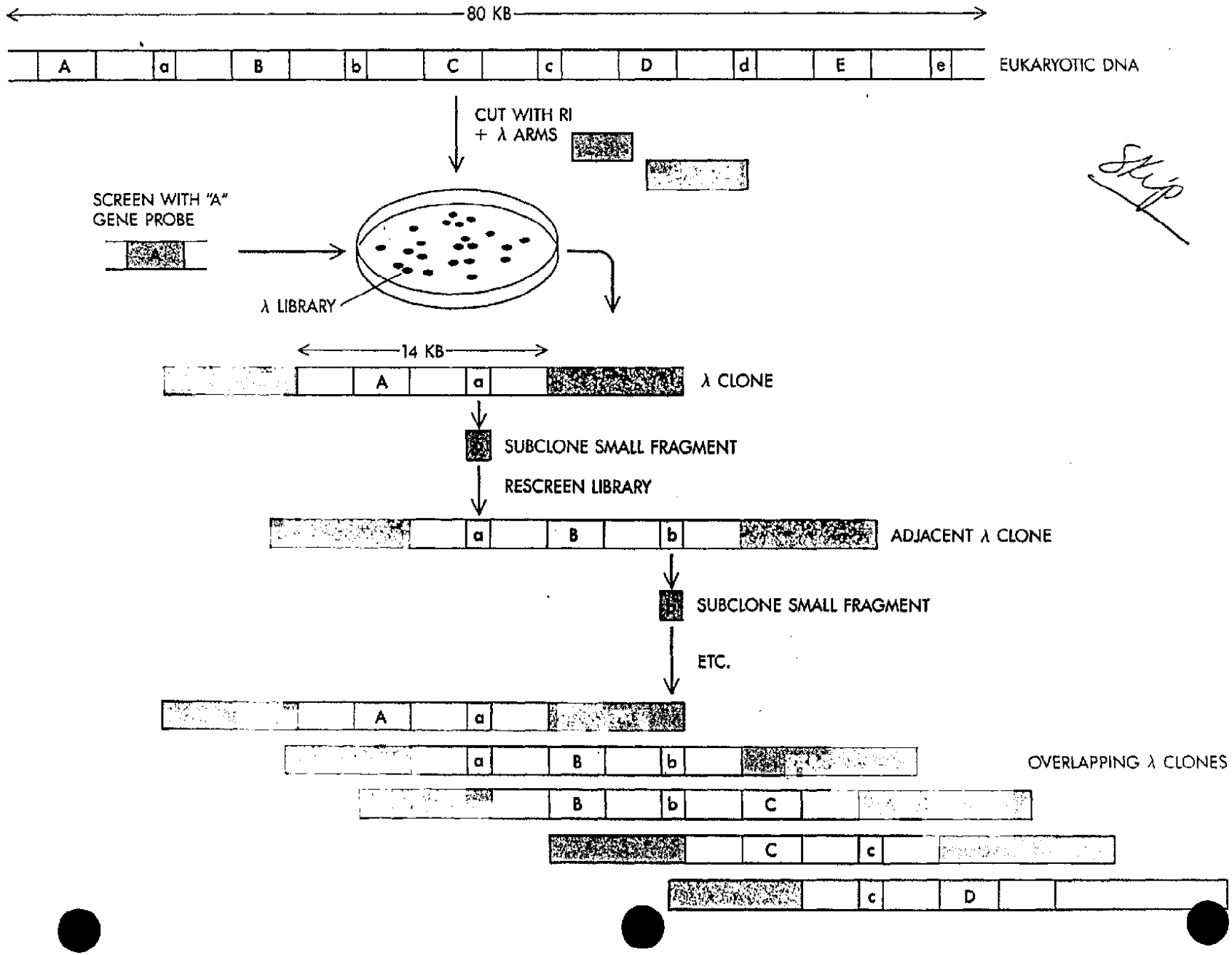
Robot to do + controlling computer = $30,000

machines all do Sanger sequencing (fl rather than radio)

Figure 6-12
The same synthetic oligonucleotide primer can be used for Sanger sequencing of any insert in M13. All insertions of foreign DNA into the RF of M13 are made at the same site in the RF. The sequence adjacent to the foreign DNA is therefore always the same. An oligonucleotide complementary to this adjacent M13 sequence can be used as a primer to sequence any piece of foreign DNA cloned into M13.

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 83- IF A NEIGHBOR OF THE DESIRED SEQUENCE HAS ALREADY BEEN CLONED, THE CORRECT CLONE CAN BE ISOLATED BY "CHROMOSOME WALKING."



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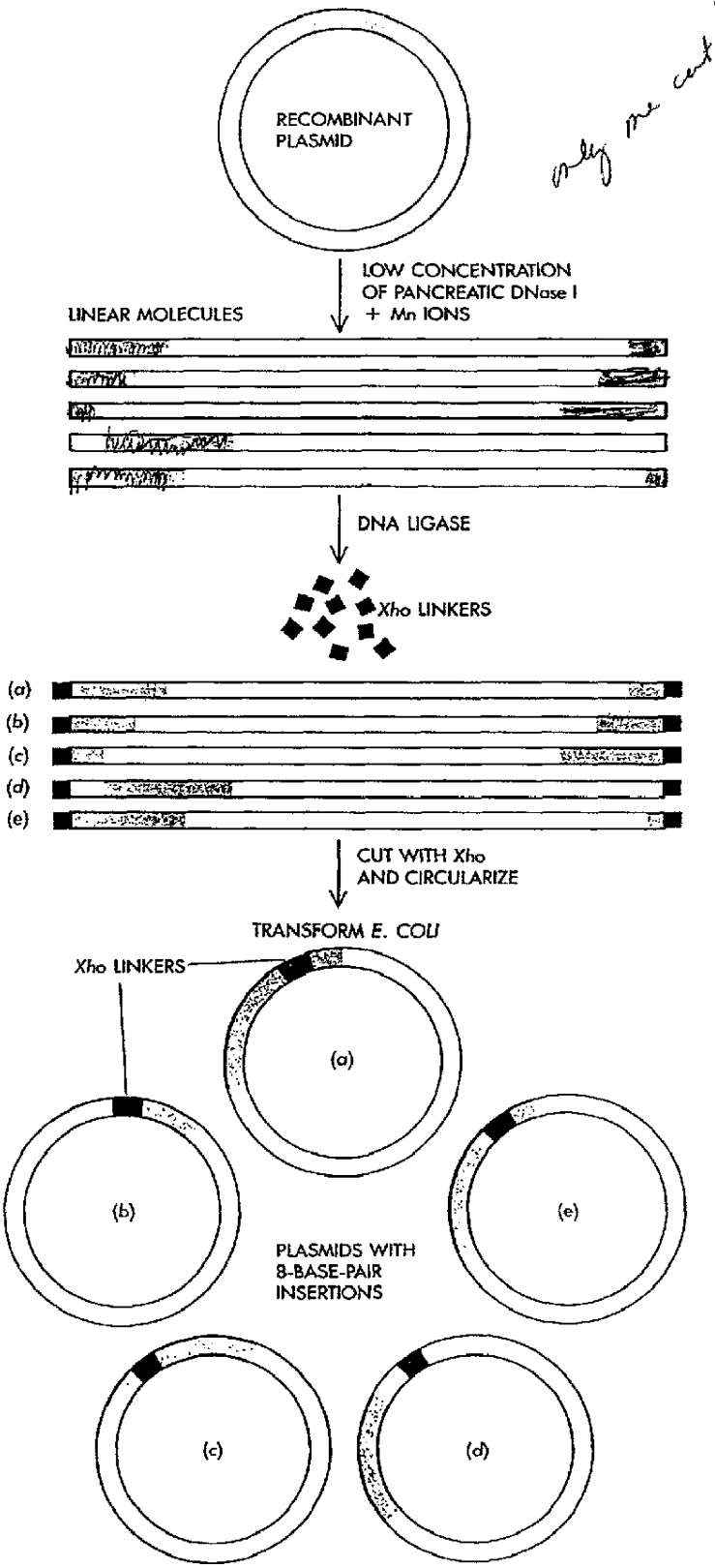
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 34- RANDOMIZED INSERTION MUTAGENESIS.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 85a- GENERATION OF A NESTED SET OF DELETIONS.

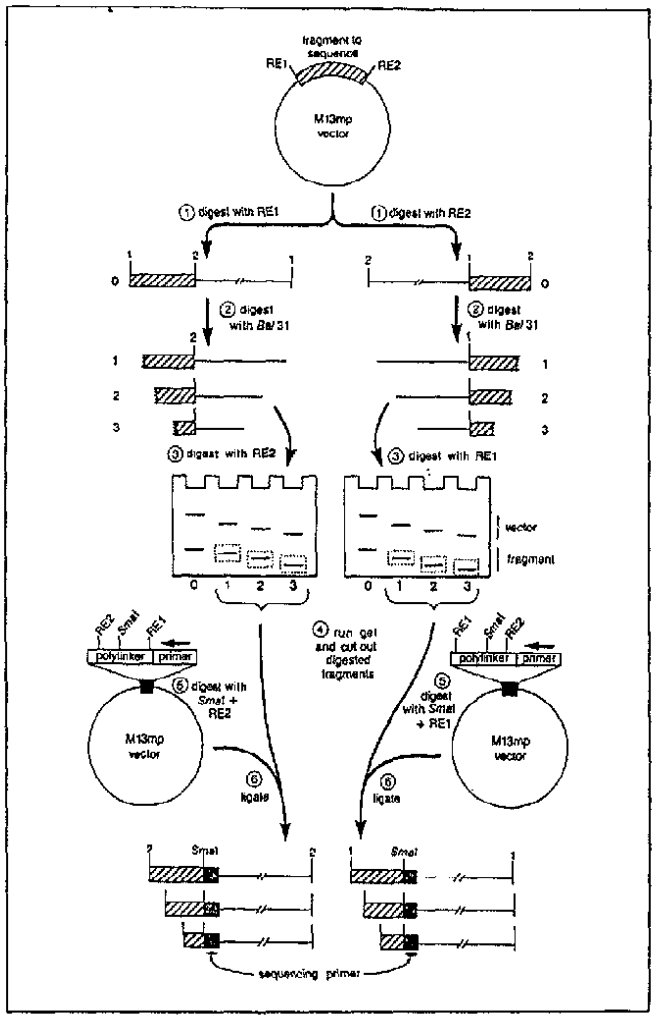
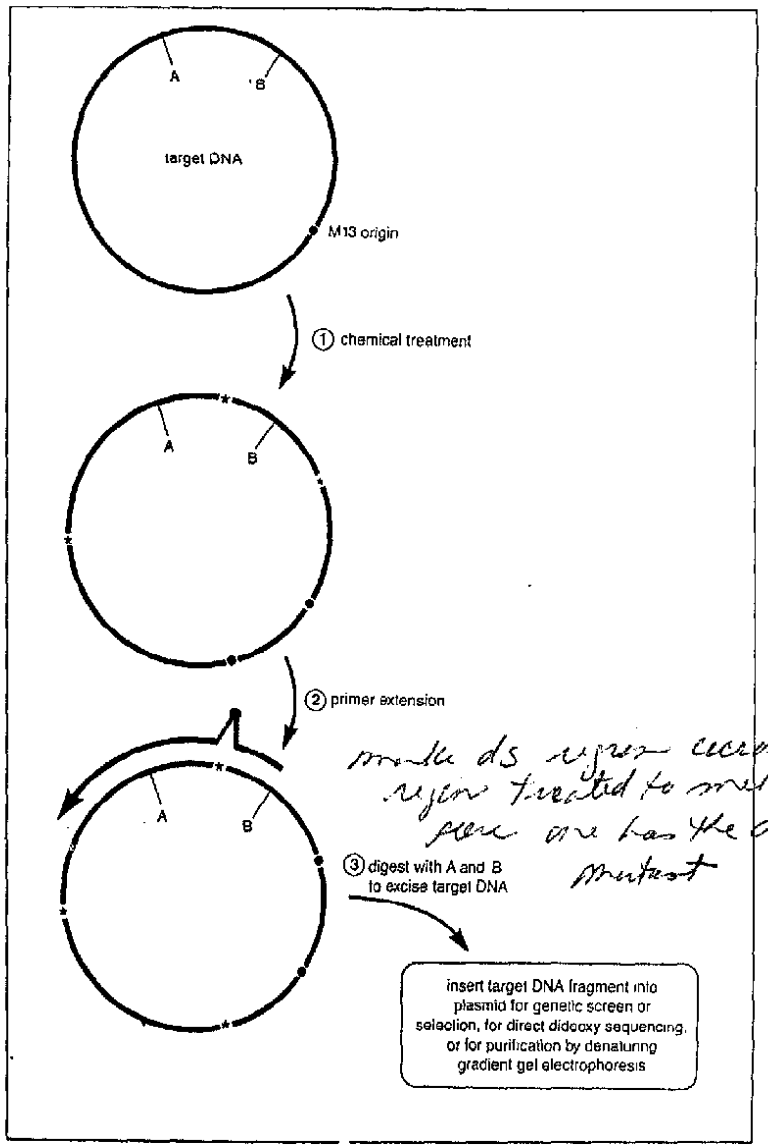


Figure 7.3.1 Strategy for making a nested set of deletions with *Bal* 31 exonuclease. The double-stranded RF form of an M13mp vector containing a fragment to be sequenced is linearized at both ends of the fragment in separate reactions (step 1) and a nested set of deletions is generated with *Bal* 31 exonuclease (step 2). The vector and insert sequences are then separated form each other by restriction endonuclease digestion (step 3) and fractionated by agarose gel electrophoresis (step 4). The digested inserts are eluted from the gel and ligated to a linearized M13mp vector (steps 5 and 6). "RE1" and "RE2" refer to two different restriction endonucleases or their respective recognition sites.

FIG 85b- LOCALIZED RANDOM CHEMICAL MUTAGENESIS.



make ds region except region treated to make sure one has the desired mutant

Figure 8.3.1 Region-specific mutagenesis by chemical treatment, primer extension, and cloning.

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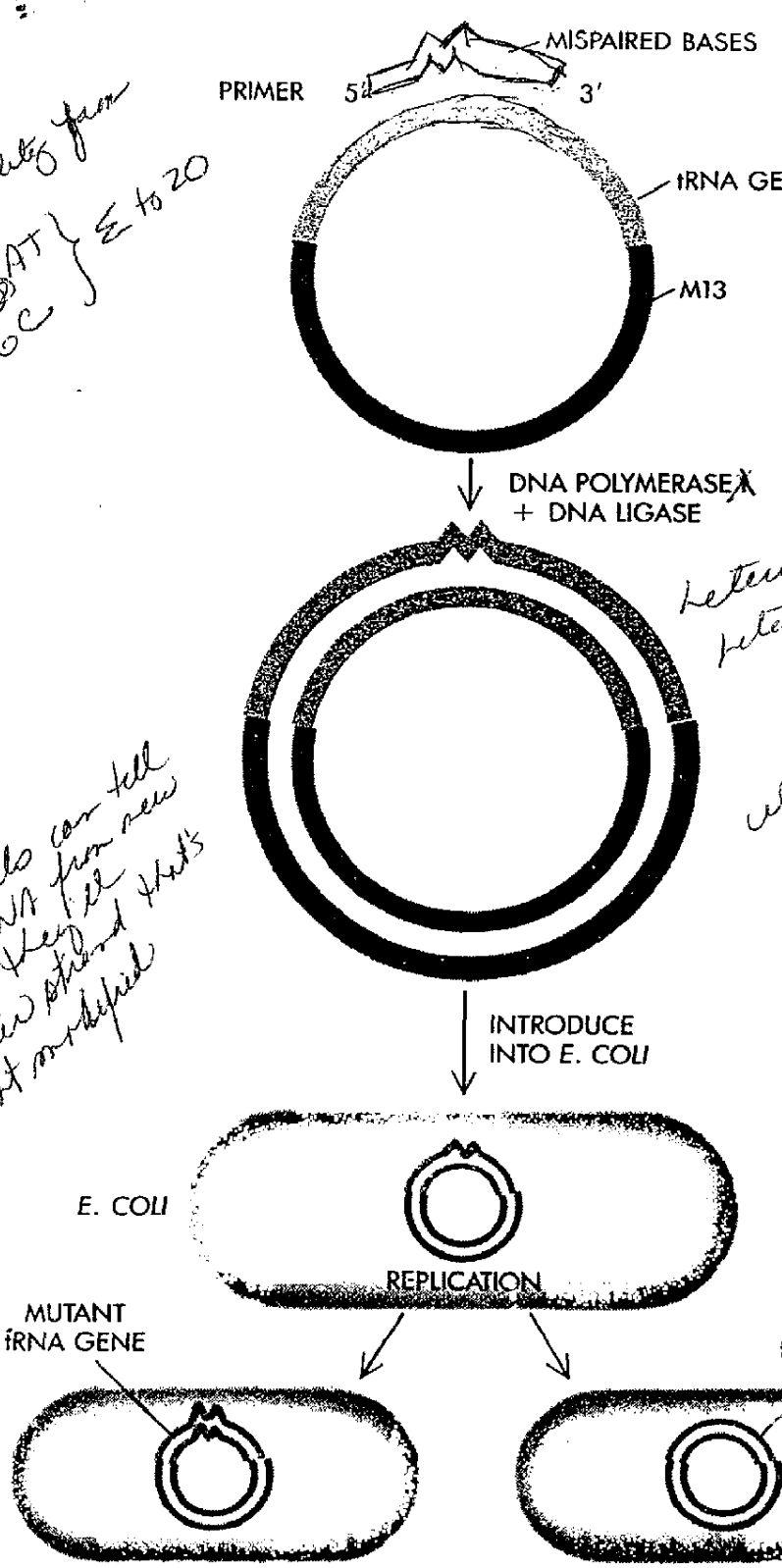
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 86- SYNTHETIC OLIGONUCLEOTIDE SITE SPECIFIC MUTAGENESIS.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

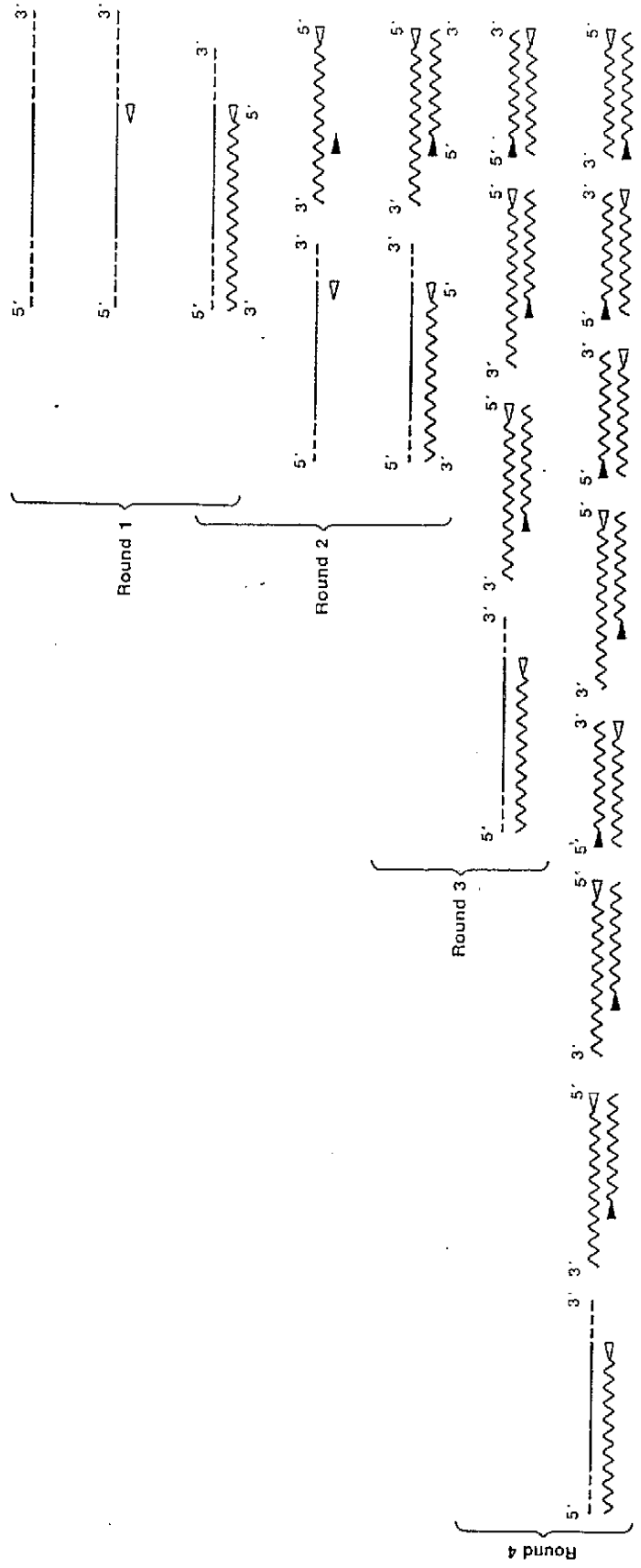
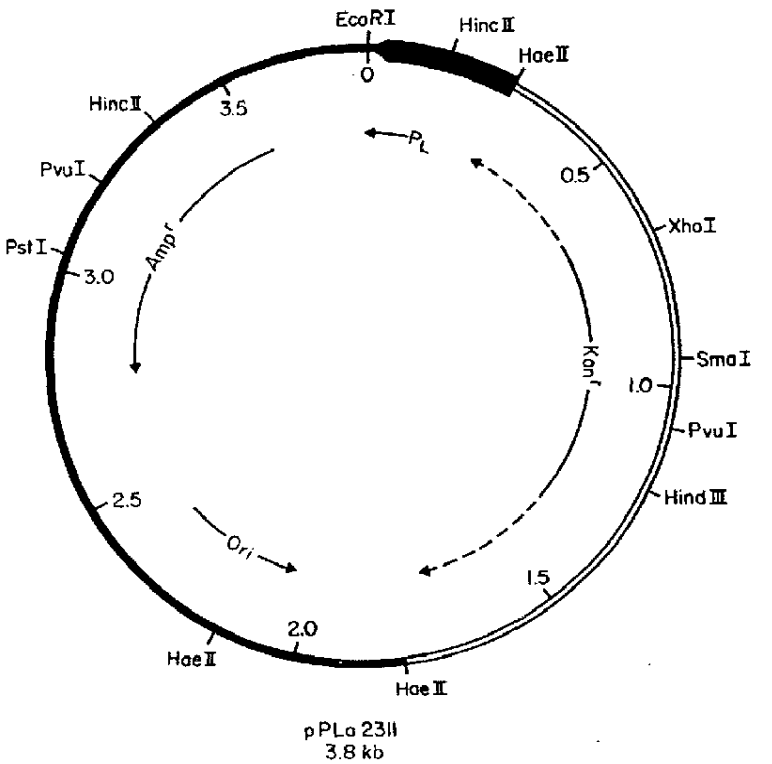


Fig 37

The diagram shows the steps involved in the first few rounds of a polymerase chain reaction. The original template (top line) is single-stranded DNA and the leftward and rightward oligonucleotide primers are shown as ◁ and ▶, respectively. The products of the first few rounds of the amplification reaction are heterogeneous in size. However, the tract of DNA lying between the two primers is preferentially amplified and quickly becomes the dominant product of the amplification reaction. If the original template is double-stranded, an equivalent set of reactions will take place using the complementary strand as template.

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 88- EXPRESSING A FOREIGN GENE IN E. COLI REQUIRES THAT THE CORRECT CODING SEQUENCE (NOT INTERRUPTED EUKARYOTIC GENOMIC DNA) BE PLACED EITHER IN FRAME WITH AN EXPRESSED VECTOR CODING SEQUENCE (TO MAKE A FUSION PROTEIN) OR IMMEDIATELY ADJACENT TO THE CORRECT TRANSCRIPTION AND TRANSLATION INITIATION SIGNALS.



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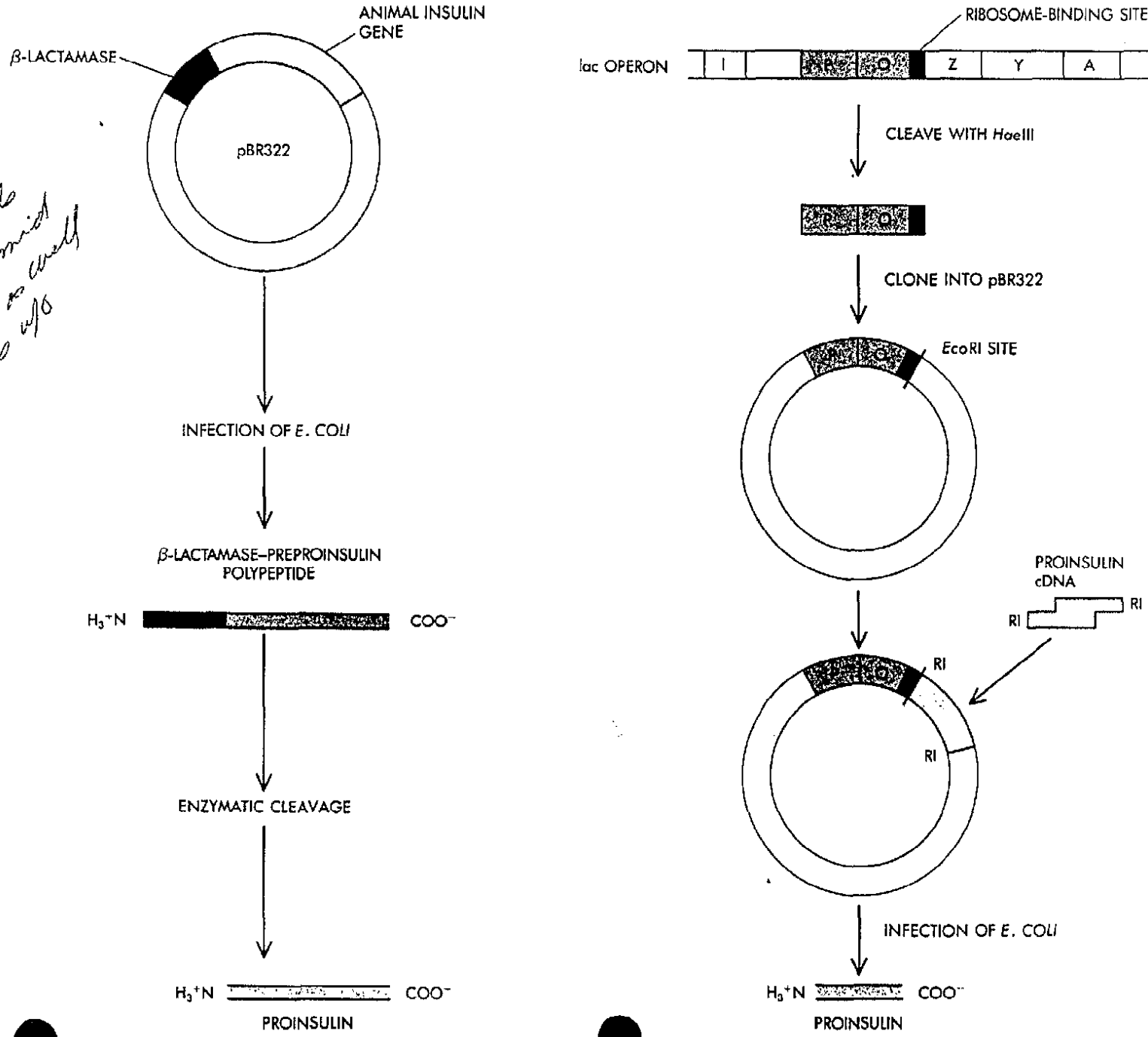
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 89- CREATING AN EXPRESSION SYSTEM FOR PROINSULIN.



want cells of plasmid to grow as well as cells w/o

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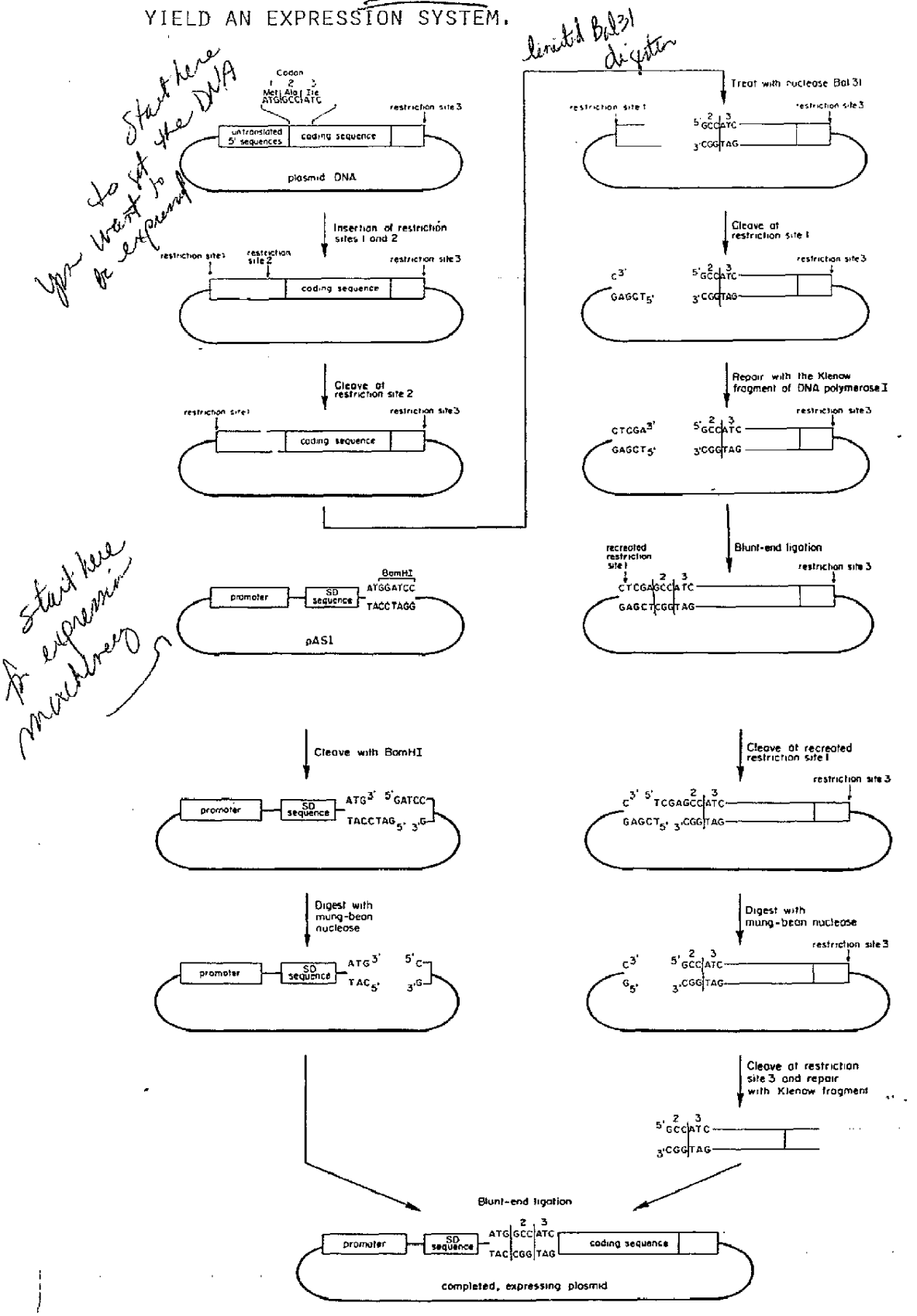
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 90- A SPECIFIC EXAMPLE OF A SERIES OF CONSTRUCTION STEPS TO YIELD AN EXPRESSION SYSTEM.



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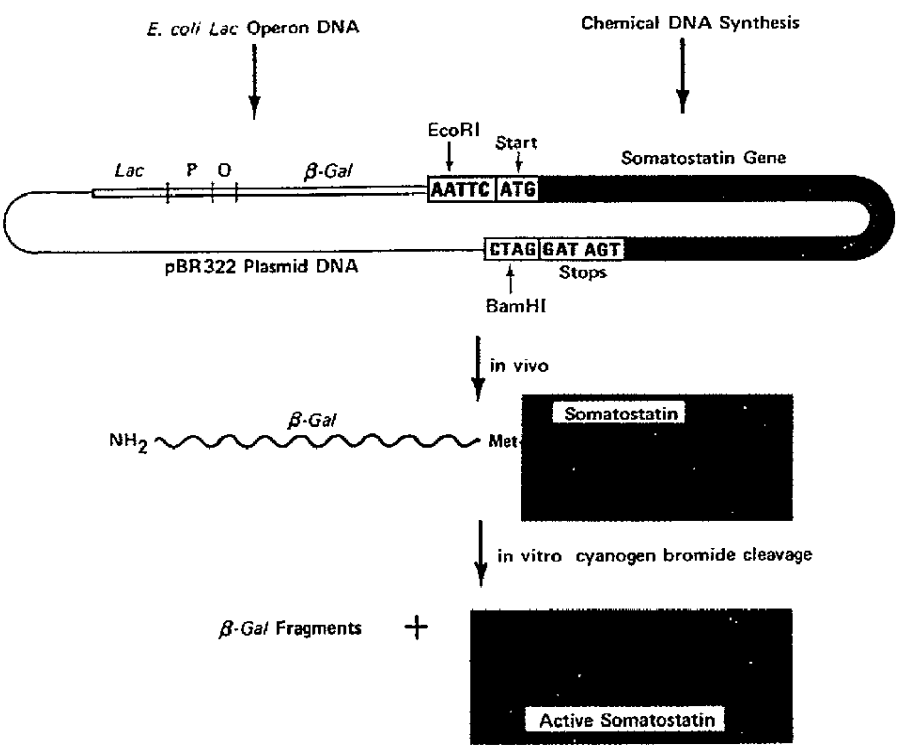
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

IF YOU DON'T GET EXPRESSION;

(1) "MINDLESSLY" TRY OTHER EXPRESSION SYSTEMS.
(2) STABILIZE PRODUCT; USE HOST MUTANTS WHICH LACK PROTEASES OR MAKE A FUSION PROTEIN.
(3) CHECK FOR INEFFICIENT TRANSLATION INITIATION (EG. mRNA 2° STRUCTURES)
(4) CHECK FOR mRNA INSTABILITY.
(5) CHECK FOR CODON USAGE PROBLEMS.
(6) USE GENETIC SELECTIONS FOR INCREASED EXPRESSION.

FIG 91- A FUSION PROTEIN EXPRESSION SYSTEM.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

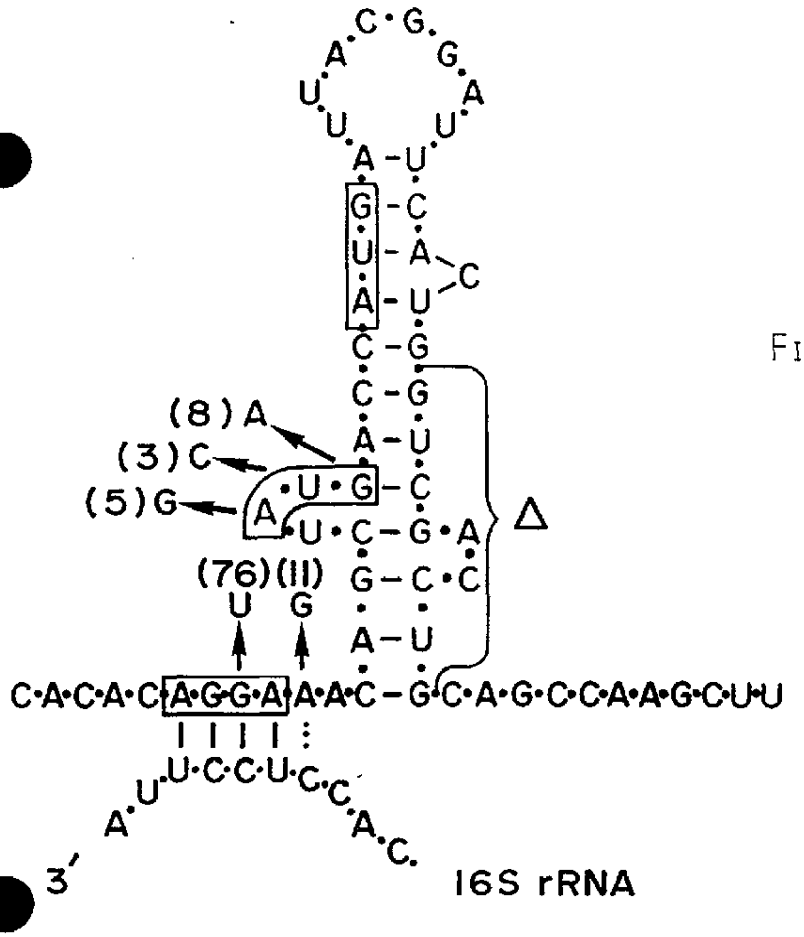
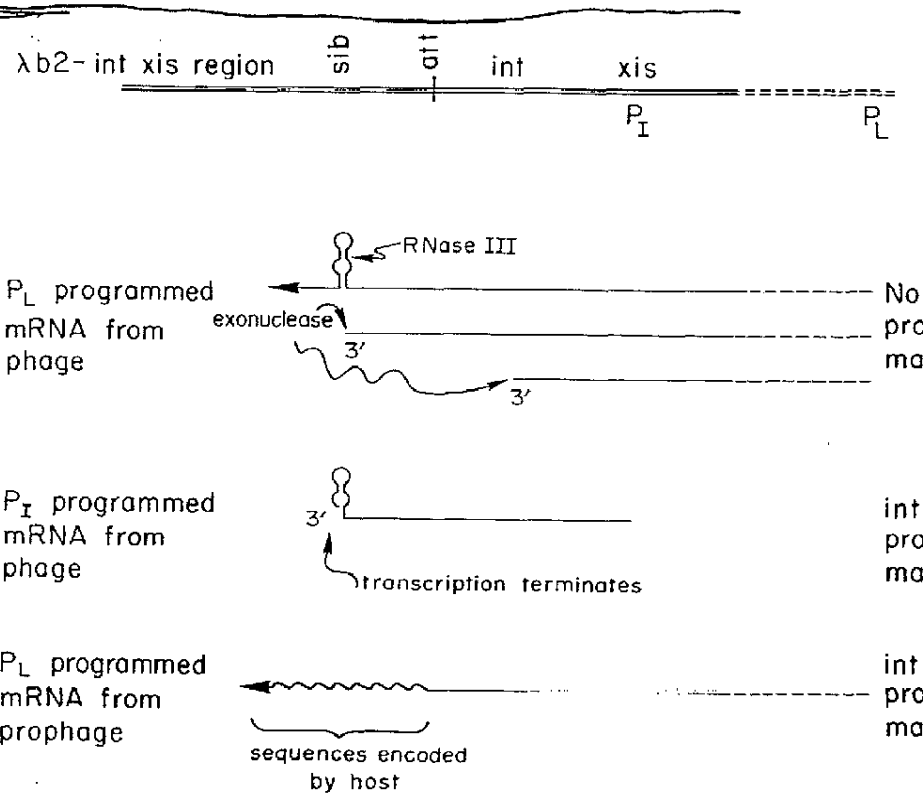


FIG 92A- SOME EXPRESSION CONSTRUCTS DO NOT WORK BECAUSE OF THE EXISTANCE OF mRNA SECONDARY STRUCTURES WHICH TIE-UP THE TRANSLATION INITIATION SIGNALS.

FIG 92B- mRNA INSTABILITY CAN ALSO CAUSE INEFFECTIVE ESPRESSION CONSTRUCTS. SECONDARY STRUCTURES AT THE mRNA 3′ END CAN STABILIZE mRNA's.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 93- TECHNIQUES USED IN ANIMAL GENETIC ENGINEERING.

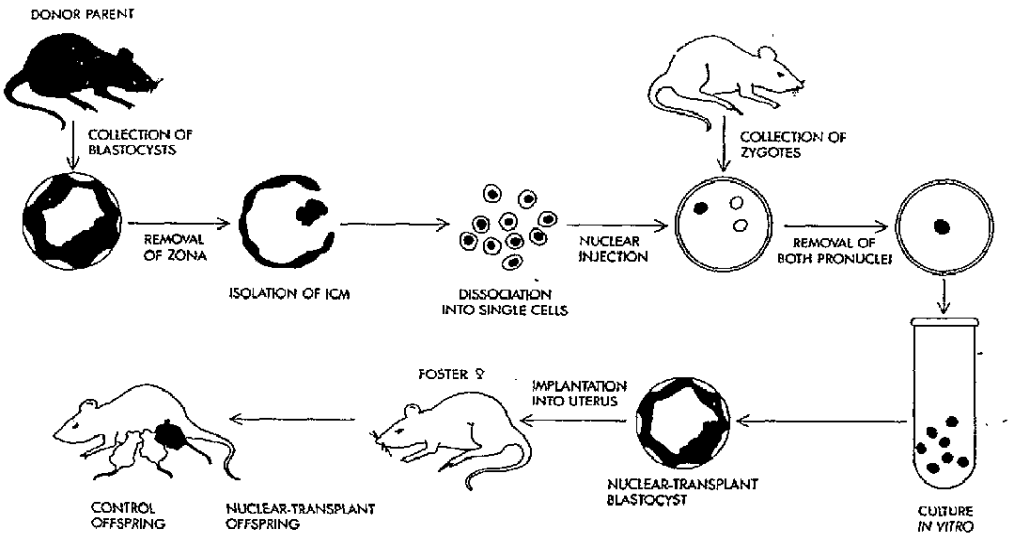

Figure 16-10
A potential scheme for cloning mice by nuclear transplantation. Totipotent mouse embryo nuclei must be collected from a pregnant female mouse. Blastocysts are obtained, and nuclei are removed from these cells by using a micropipette and injected into another fertilized mouse egg. The genome (the male and female pronuclei) of the recipient egg is then removed. The transplanted embryos are cultured until the blastocyst stage and then implanted into foster mothers.

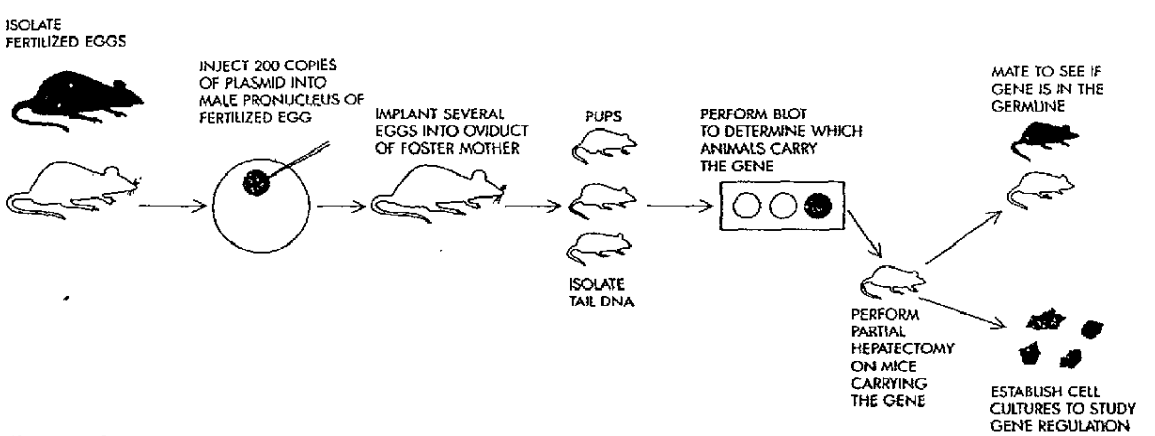

Figure 16-1
Microinjection of cloned genes into mouse embryos.

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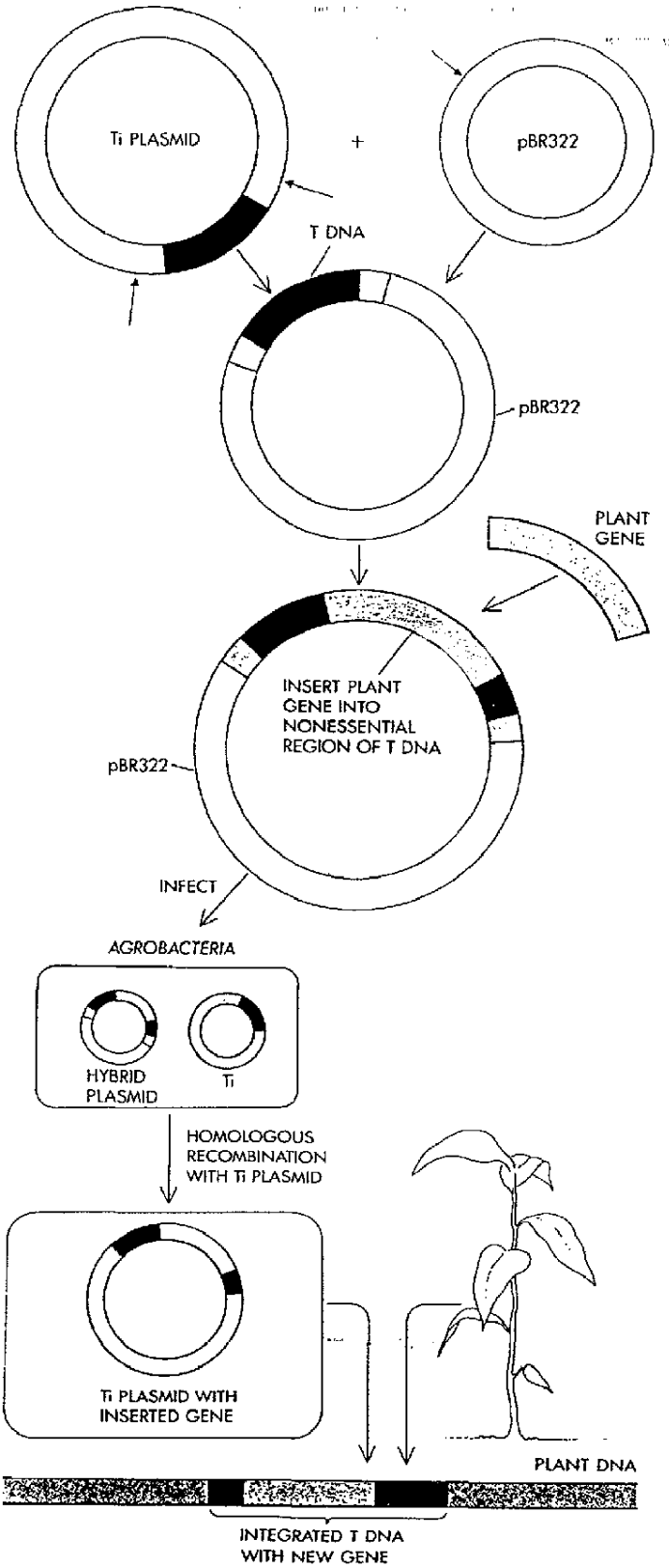
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 94--THE USE OF Ti PLASMIDS TO INTRODUCE FOREIGN DNA INTO PLANTS.



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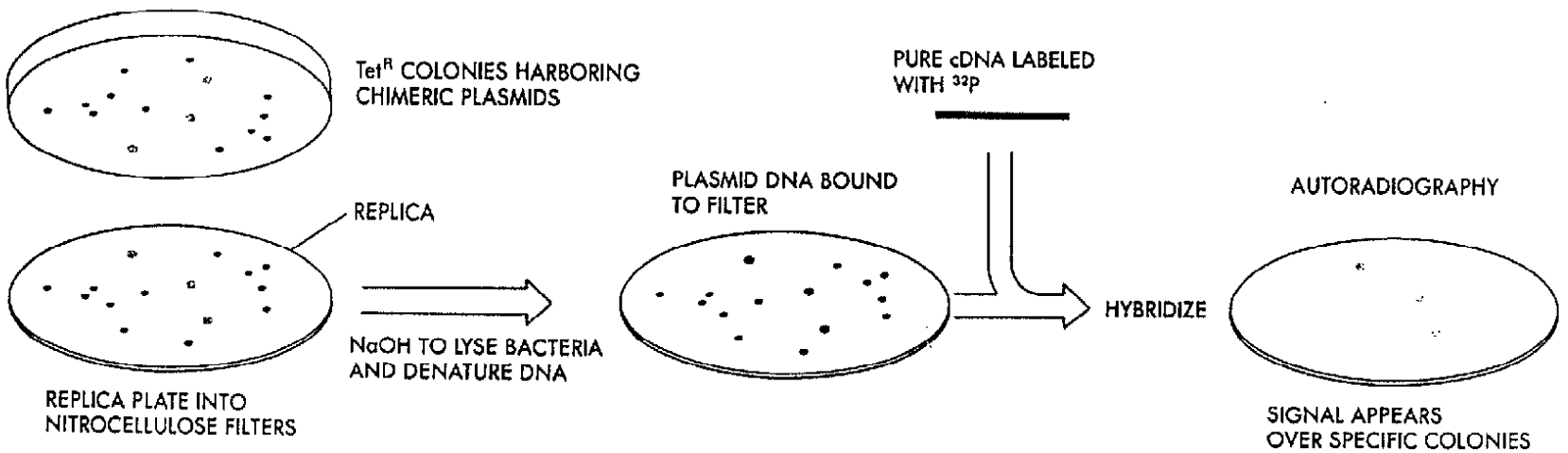
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 95- FILTER HYBRIDIZATION OF COLONY COPIES CAN BE USED TO IDENTIFY THE ORGANISM FORMING THE COLONY.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG 96- SOME GENETIC DISEASES ALTER RESTRICTION DIGEST PATTERNS AND CAN BE DETECTED WITH SOUTHERN BLOTS.

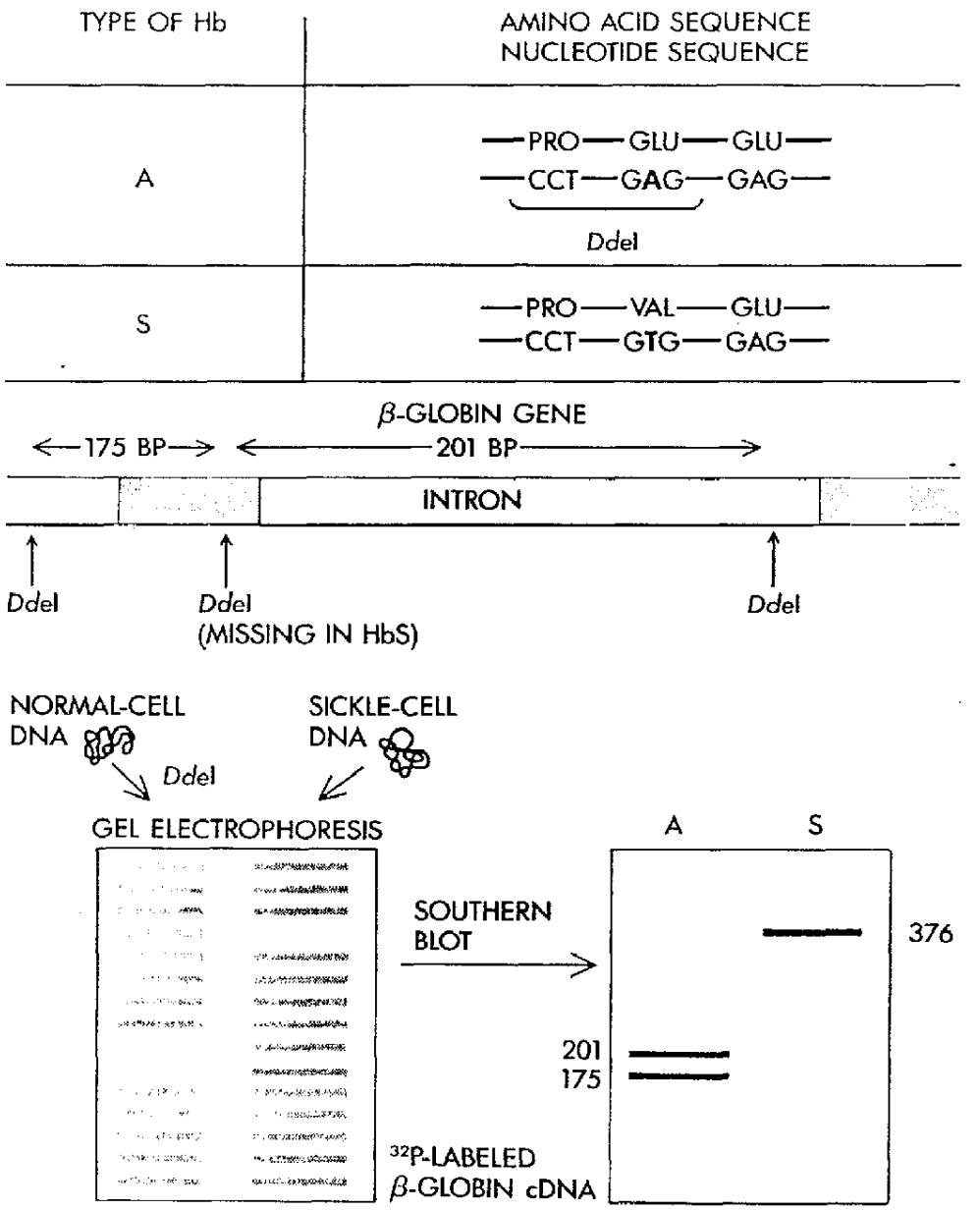


Figure 17-2
Detection of the sickle-cell globin gene by Southern blotting. The base change (A → T) that causes sickle-cell anemia destroys a *Dde*I site that is present in the normal β-globin gene. This difference can be detected by Southern blotting.

Can detect by the restriction site

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

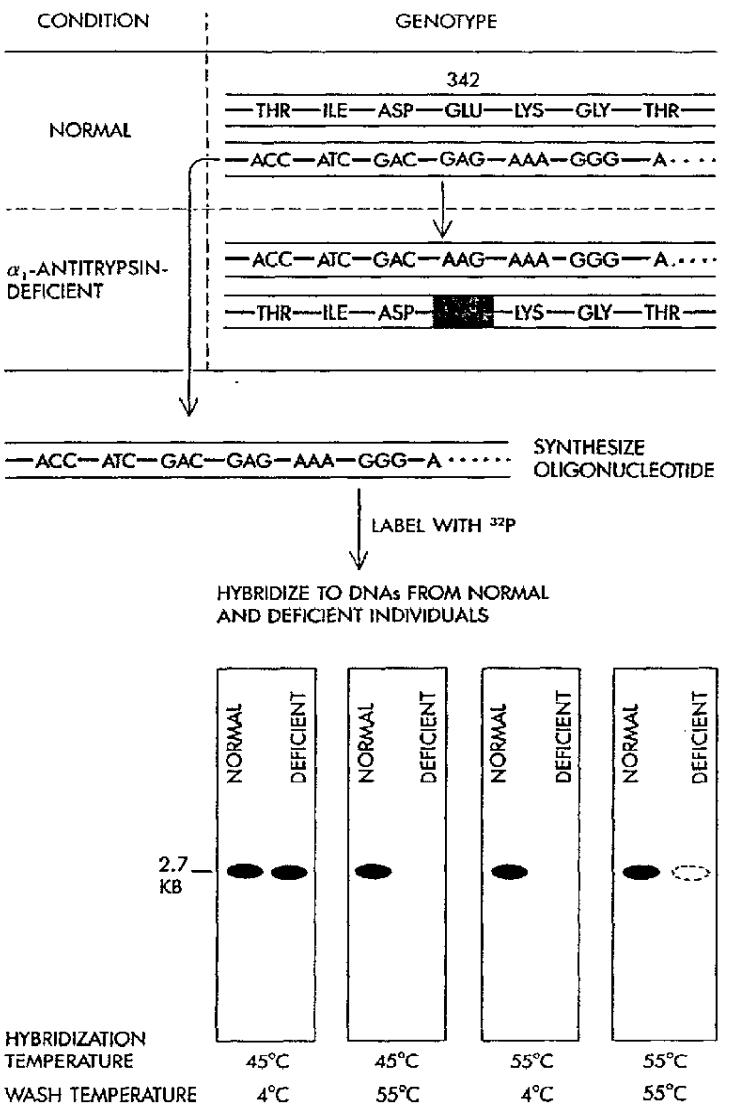


Figure 17-3
Diagnosis of α_1-antitrypsin deficiency by using a synthetic oligonucleotide. DNA from a patient with α_1-antitrypsin deficiency was analyzed and found to have a single base change (G → A) in the protein-coding region. A 19-base oligonucleotide was synthesized chemically to be complementary to the normal gene (and therefore to have one mismatch with the mutant gene). This oligonucleotide can distinguish between the normal and mutant genes when it is used as a probe in Southern blot analysis (Chapter 6).

FIG 97- ALL GENETIC DISEASES ALTER DNA SEQUENCES. THE RELEVANT CHANGE, IF IN A UNIQUE SEQUENCE, CAN BE DETECTED BY VIRTUE OF THE DESTABILIZATION EFFECT OF BASE PAIR MISMATCHES.

PM3001193176

Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG. 107: GENE CLONING BEGINS WITH AN UNDERSTANDING OF THE DESIRED PROTEINS' PHYSICAL PROPERTIES, SITE OF SYNTHESIS, ASSAY AND LIKELY ACTIVITIES.

AA sequence known for its entirety

WHAT IS SMC?

-70 amino acid protein with strong homology to proinsulin however, the central "C" peptide is not processed. somatomedin-C is identical to insulin-like growth factor I

-traditionally purified from human serum where it can be detected by RIA at 0.2-0.5 ug/ml, bound to a carrier protein.

-shown to be synthesized in liver by in vitro rat liver perfusion and mRNA cloning.

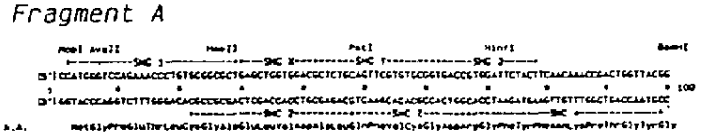
-has a receptor which is similar but distinguishable from the insulin receptor, to which it will bind at 100x higher concentrations than for insulin.

-a growth factor, able to replace growth hormone in the hypophysectomized rat tibia assay.

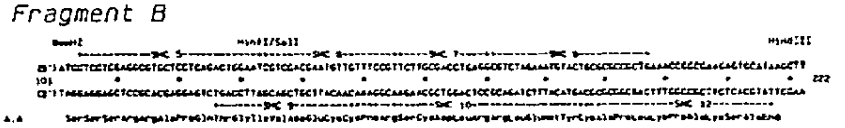
FIG. 108: SYNTHETIC GENES FOR SMALL PROTEINS CAN BE CONSTRUCTED BY ORGANIC CHEMISTRY TECHNIQUES. COMPONENTS ARE SYNTHETIC SINGLE-STRANDED FRAGMENTS OF 20 - 40 BASE PAIRS.

H_2O competes in cyclization; must make ~100 μg to compute appropriately w/ humidity & loss

Fragment A



Fragment B



codon degeneracy — divide based on what host uses most freq. for a given AA

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG. 109: EXPRESSION PLASMIDS FOR E. COLI ARE ENGINEERED FROM ELEMENTS OF BACTERIOPHAGE AND TRANSPOSONS.

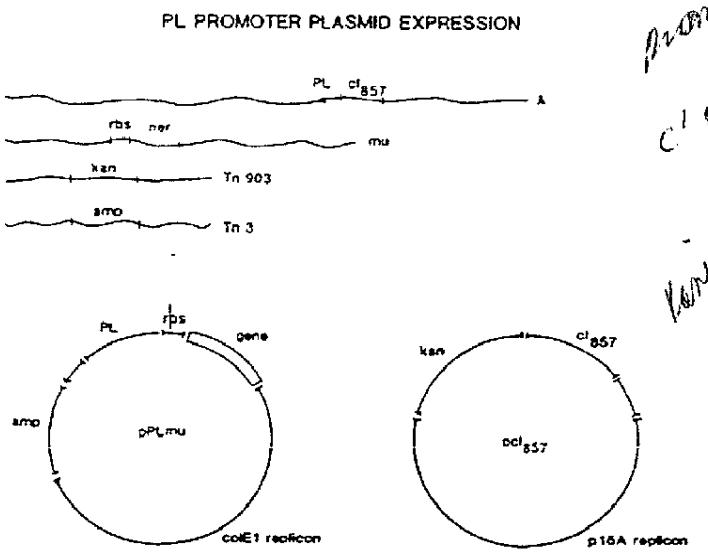
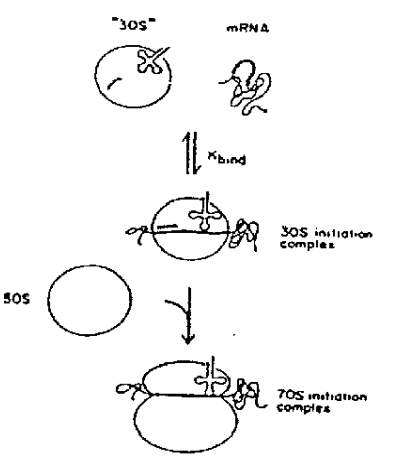


FIG. 110: INDUCTION OF PLASMID EXPRESSION RESULTS IN THE SYNTHESIS OF MRNA TO WHICH THE 30S RIBOSOMAL COMPONENT BINDS FOR TRANSLATION INITIATION.



Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG. 111: RNA "STEM AND LOOP" SECONDARY STRUCTURES IN THE REGION OF RIBOSOME-BINDING ARE CORRELATED WITH mRNAs WHICH PRODUCE LITTLE OR NO PROTEIN.

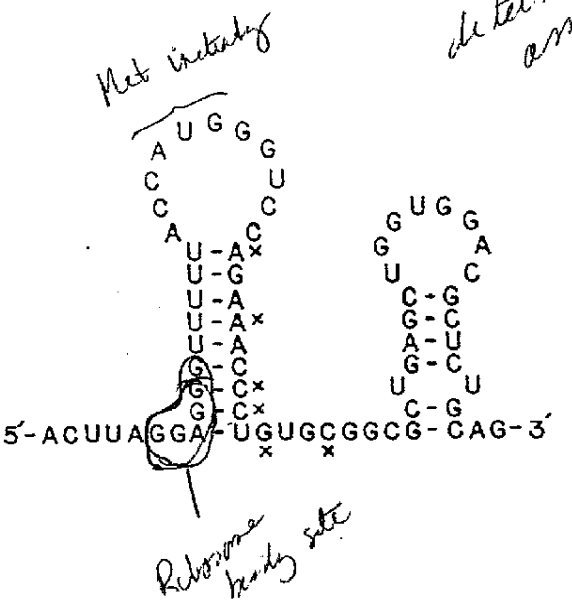


FIG. 112: MANY EXPERIMENTAL CLONING PROBLEMS ARE ADDRESSED WITH pUC PLASMIDS. THEIR UTILITY IS DERIVED FROM THE INSERTIONAL INACTIVATION OF LACZ AT SEVERAL UNIQUE RESTRICTION SITES.

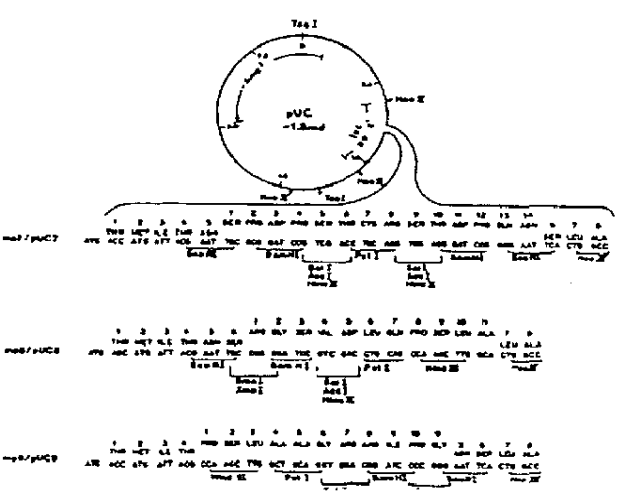


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FIG. 113: "IN FRAME" GENE FUSIONS WITH THE LACZ GENE IN pUC PLASMIDS CAN FORM FUSION PROTEINS WHICH MAY (OR MAY NOT) MAINTAIN β-GALACTOSIDASE ACTIVITY.

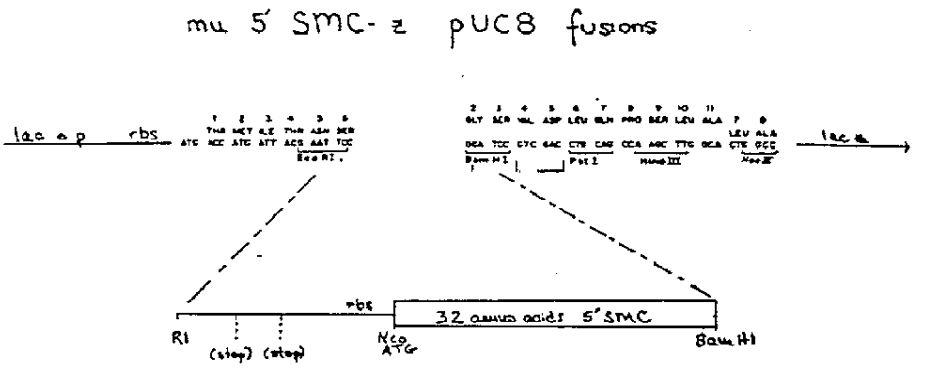
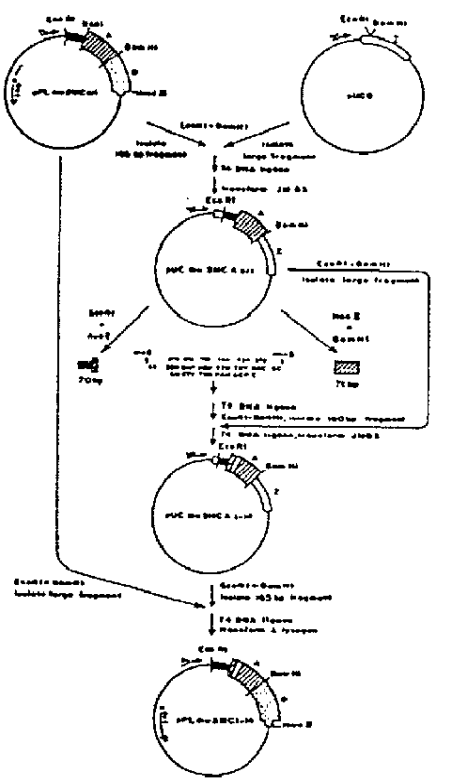


FIG. 114: "IN FRAME" LACZ GENE FUSIONS WHICH ARE LACZ⁻ IN PHENOTYPE CAN BE USED TO SCREEN FOR "UP" MUTATIONS THAT RESTORE THE ACTIVITY OF β-GALACTOSIDASE.



Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG. 115: THE LACZ PHENOTYPE OF INDIVIDUAL COLONIES CAN BE MONITORED ON PLATES CONTAINING 5-BROMO-4-CHLORO-3-INOXYL-β-D-GALACTOSIDE (X-GAL), A CHROMOGENIC SUBSTRATE FOR β-GALACTOSIDASE.

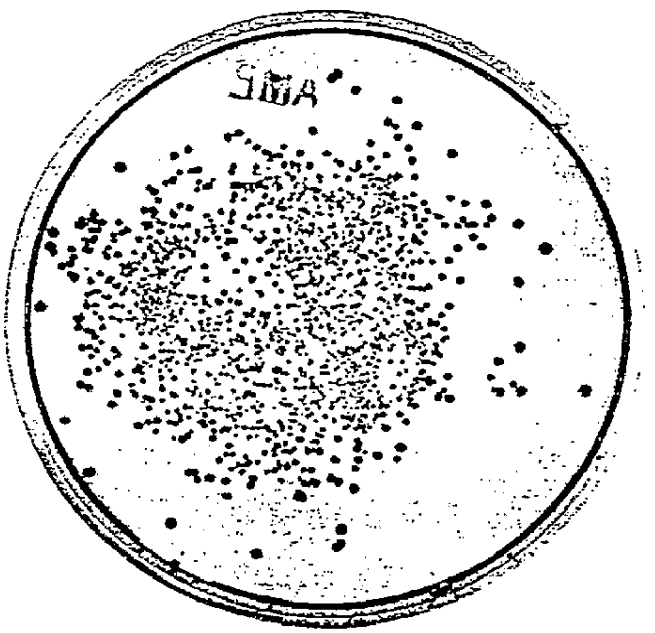
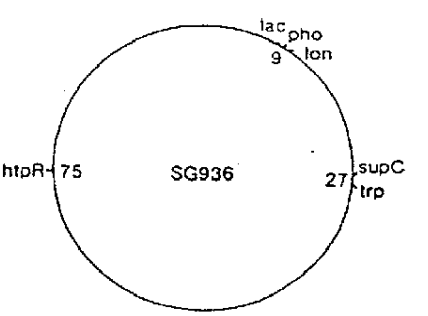


FIG. 116: OTHER "UP" MUTATIONS FOR SMALL PROTEIN ACCUMULATION CAN BE ISOLATED IN CHROMOSOMAL GENES SUCH AS LON, WHICH ENCODES A PROTEASE AND HTPR, WHICH REGULATES HEAT-SHOCK EXPRESION.

encodes an alternative σ subunit of DNA polymerase (σ32)

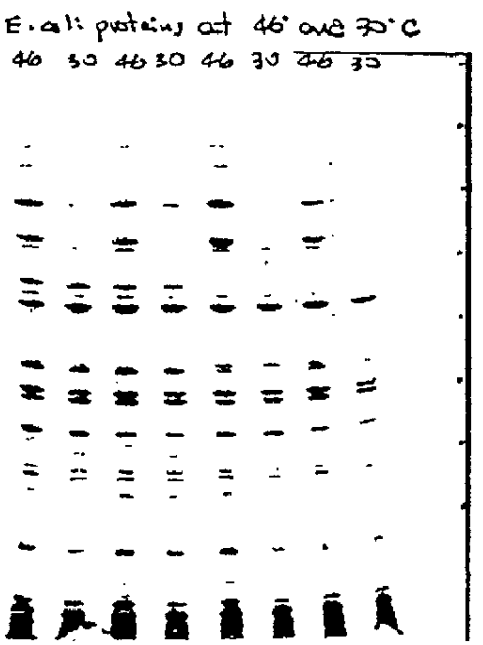
Derivation of lon htpR E. coli

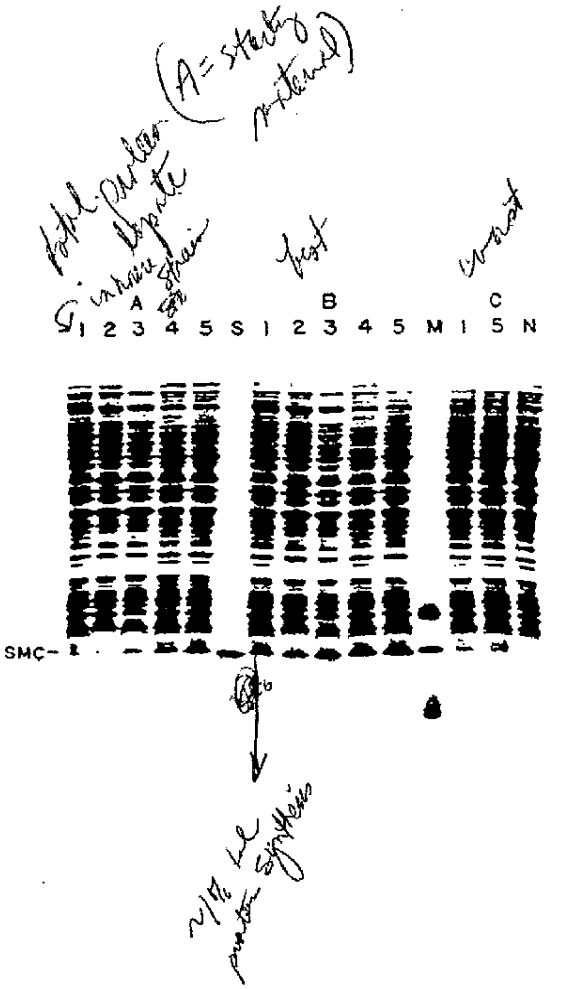


Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG. 117: MANY KINDS OF STRESS, INCLUDING RAPID TEMPERATURE INCREASE RESULT IN THE TEMPORARY INDUCTION OF "HEAT-SHOCK" PROTEINS IN E. COLI AND OTHER ORGANISMS.

FIG. 118: MUTATIONS IN LON AND HTPR BOTH ENHANCE SMC ACCUMULATION ABOUT 2-FOLD. THEIR EFFECTS ~~ARE ADDITIVE~~. Their effects are additive





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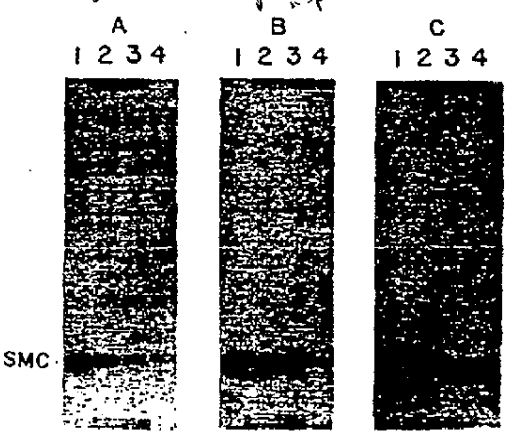
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG. 119: PULSE-CHASE EXPERIMENTS WITH RADIOACTIVE AMINO ACIDS ARE USED TO DETERMINE THE HALF-LIVES OF PROTEINS. THIS TECHNIQUE CAN DEMONSTRATE THAT THE LON + HTPR MUTATIONS INCREASE THE HALF-LIFE OF SMC IN E. COLI.

FIG. 120: A GIVEN TECHNIQUE CAN'T SOLVE ALL EXPERIMENTAL PROBLEMS. THE SMC-LACZ FUSIONS WERE USEFUL FOR FINDING "UP" MUTATIONS WITH "MU" AND "TRP" RIBOSOME-BINDING SITES, BUT NOT THAT FROM MS2.



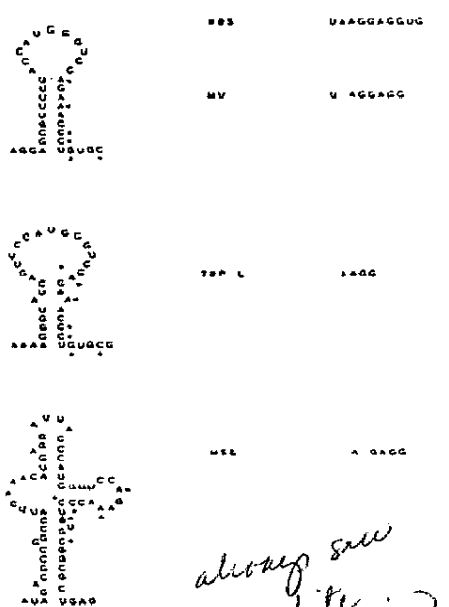


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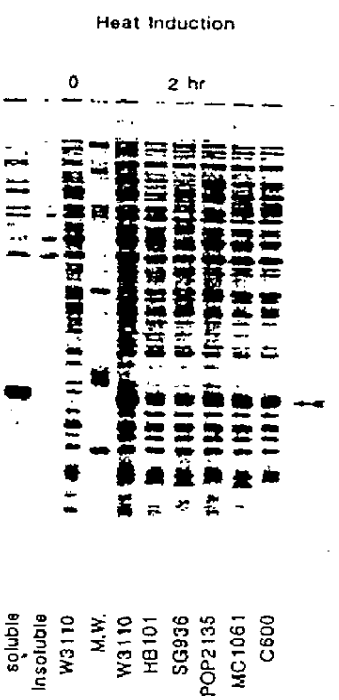
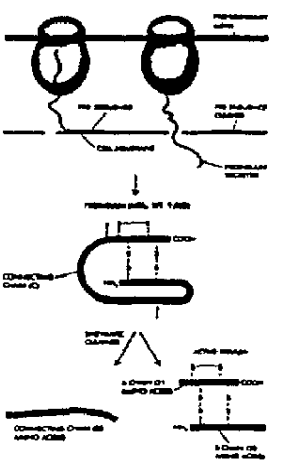
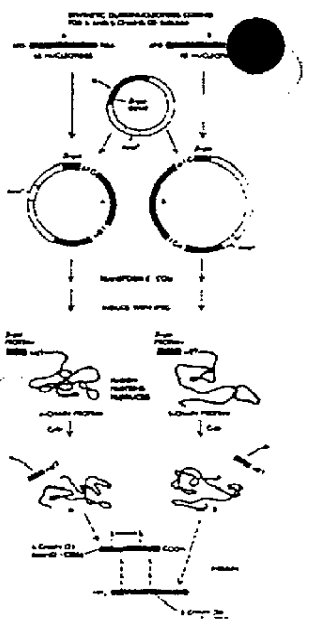
FIG. 121: THE LON + HTPR MUTANT STRAIN SG936 WAS THE BEST PRODUCER OF SMC AND THE WORST PRODUCER OF A DIFFERENT EUKARYOTIC HORMONE G-CSF.

FIG. 122: ANOTHER TECHNIQUE FOR PRODUCING SMALL UNSTABLE PROTEINS IS TO EXPRESS FUSION PROTEINS WHICH ARE CHEMICALLY CLEAVED AFTER PURIFICATION. THIS IS A COMMERCIAL TECHNIQUE FOR INSULIN PREPARATION.

STRAIN DEPENDENCE of hG-CSF PRODUCTION



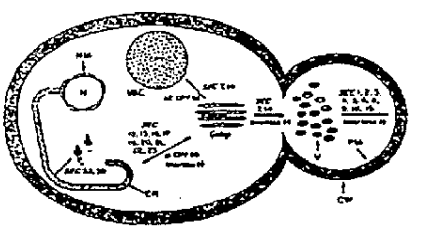


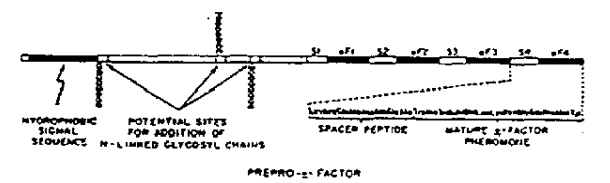


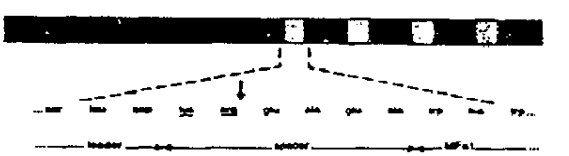
Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

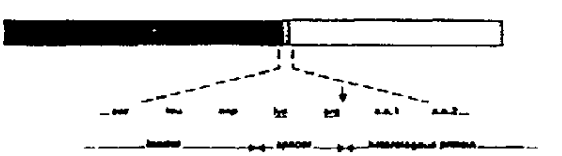
FIG. 123: RECOMBINANT PROTEINS ARE ALSO PRODUCED BY SECRETION FROM YEAST WHICH ARE EUKARYOTIC CELLS WITH INTRACELLULAR STRUCTURES THAT ARE MUCH MORE COMPLICATED THAN THOSE OF E. COLI.

FIG. 124: PROCESSING AND SECRETION SIGNALS FOR A NORMALLY SEPARATED YEAST PROTEIN, α-MATING FACTOR, ARE OFTEN EMPLOYED IN RECOMBINANT DNA CONSTRUCTS TO SIGNAL SECRETION OF THE DESIRED HETEROLOGOUS PROTEIN.









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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

FIG. 125: BECAUSE HUMAN GENOMIC DNA CONTAINS MANY SEQUENCES (HIGH COMPLEXITY), CLONING A SPECIFIC HUMAN GENE REQUIRES SEARCHING AMONGST 10^6 RECOMBINANTS, EACH WITH 20 KB OF HUMAN DNA.

Construction of Representative Genomic DNA Libraries

Table 1. The Number (N) of Independent Recombinant DNA Molecules that must be Recovered in either a Lambda Replacement Vector, or a Cosmid, in order to have a 99% Probability of having Cloned a Particular DNA Sequence.

Organism	*Approximate DNA content of haploid genome (bp)*	*N for 20 kb inserts*[a]	*N for 45 kb inserts*[a]
Escherichia coli (bacterium)	4.2×10^6	9.2×10^5	4.3×10^5
Saccharomyces cerevisiae (yeast)	1.4×10^7	3.2×10^6	1.4×10^6
Drosophila melanogaster (fruit fly)	1.4×10^8	3.2×10^7	1.4×10^7
Strongylocentrotus purpuratus (sea urchin)	8.6×10^8	2.0×10^8	8.8×10^7
Homo sapiens (man)	3.3×10^9	7.6×10^8	3.4×10^8
Triticum aestivum (hexaploid wheat)	1.7×10^{10}	3.9×10^9	1.7×10^9

[a]The calculations were based on the assumption that cleavage of the donor DNA occurs at random, which will not be strictly true for DNA cleaved by partial digestion with *Sau*3A or *Mbo*I. The numbers are likely, however, to be a sufficient approximation for most purposes.

FIG. 126: THE 20 KB HUMAN DNA PER INDIVIDUAL RECOMBINANT IS A CONSEQUENCE OF THE MAXIMAL INESSENTIAL REGION IN λ.

K. Kaiser and N. Murray

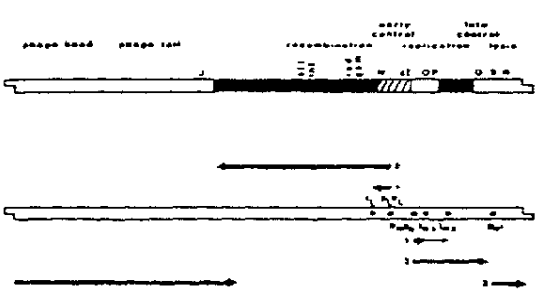

Figure 6. (a) Genes which promote related functions are clustered on the lambda genome. The block regions are inessential for propagation of lambda, and have been deleted during constructions of the vectors described here (*Figure 8*). The bracketed box is also unessential if the recombination sites $b_{1/2}$ (see below) is absent. The *cI* gene, encoding the lambda repressor, is deleted from the vectors described in this chapter, so both the vectors and their recombinant derivatives are obliged to evaluate on a lysic growth pathway (*Figure 7*). (b) Transcription of the genome proximate mainly from the promoters, p_L and p_R. Early during infection transcripts initiated at p_L and p_R (1) terminate at t_L and t_{R1} respectively (some rightward transcripts escape termination at t_{R1} and reach t_{R2}). The allowed synthesis of the *N* gene product, in the presence of which transcription may proceed through t_L and t_{R1} into adjacent genes (2). This in turn allows synthesis of the *Q* gene product in the presence of which "late" transcription (3), initiated from p_R, ... through the lysis genes, past the cos site (of the circular genome; *Figure 7*), through the ... genes, and past the tail fibre gene *J*. That gene *N* (and its product) are normally essential for ... substrates. If t_{R1} is deleted, however, *N*-independent "leakage" of transcription through t_{R1} ... Q functions. Most lambda vectors, including those described here, isolate cI857 spontaneously ... time (week) then removes t_{R1}. Thus deletions within *N* and the p_L promoter responsible for phage growth, however, the vectors described in *Figure 8*, and their recombinant derivatives, are *N*$^-$ so that they do not have to rely on transcriptional leakage and, therefore, grow relatively vigorously. (−) major promoters; (+) major termination signals.

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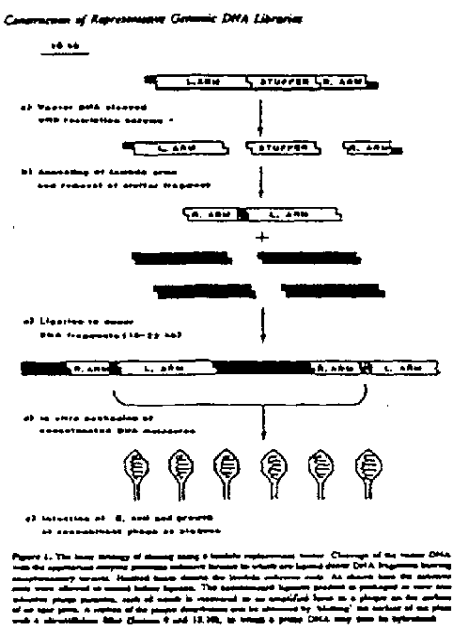
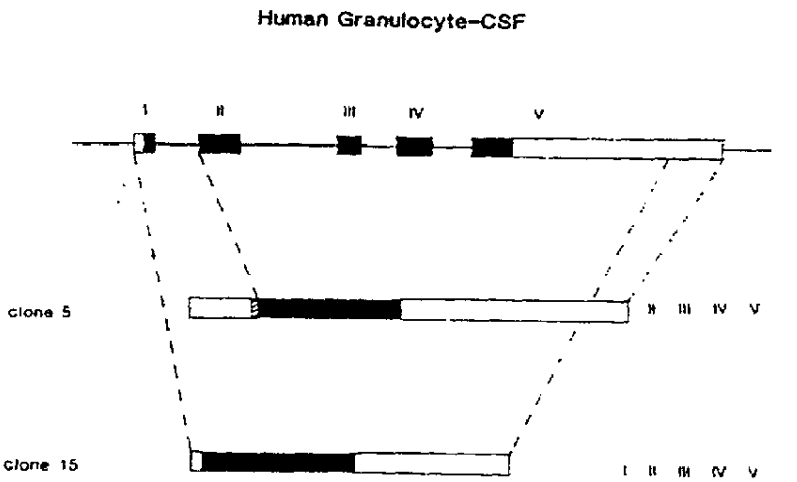
SKIP

FIG. 127: THE ESSENTIAL REGIONS OF λ ARE COMPOSED OF A 20 KB "LEFT ARM" WITH GENES FOR PHAGE MATURATION AND A 9 KB "RIGHT ARM" WITH GENES FOR PHAGE DNA REPLICATION.

FIG. 128: MOST GENOMIC GENES CONTAIN "INTRONS" - REGIONS OF DNA THAT ARE TRANSCRIBED BUT LATER EXCISED FROM THE mRNA.





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30 × 350 ≈ 100,000
100 × 230 ≈ 250,000
11,000 × 14 = ≈ 50,100
≈ 500,000

14 / 500,000 = 1 / 35,000

99% prob ⇒ plate out ≈ 160,000 colonies!

GB 12

Banks for cloning eukaryotes are ≈ 10,000,000

2 people can screen 1×10⁶ in 2 days

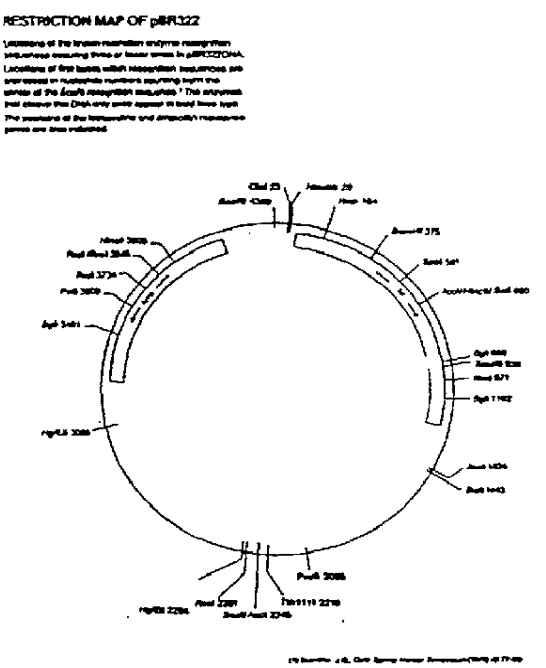
FIG. 129: SPECIFIC MRNAS VARY WIDELY IN THEIR ABUNDANCY

Table 1 The abundance and complexity of the mRNA population of a typical eukaryotic cell

Abundance class	Fraction of the mRNA population in the abundance class	Number of different mRNA sequences in the abundance class	Number of copies per cell of each different mRNA sequence
High	22%	30	3500
Medium	49%	1090	230
Low	29%	10670	14

These data derive from an analysis of the mRNA population of an SV40 transformed, human fibroblast cell line. Reprinted from Williams *et al.* (1979), with permission of the MIT press.

FIG. 130: UNTIL VERY RECENTLY CDNA - SYNTHETIC, COMPLEMENTARY COPIES OF MRNA - WERE CLONED IN PLASMID PBR322 BY THE TECHNIQUES IN FIG. 70.



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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001

Fig. 131: gt10 and gt11 are phage with unique sites for Eco RI engineered for cDNA cloning.

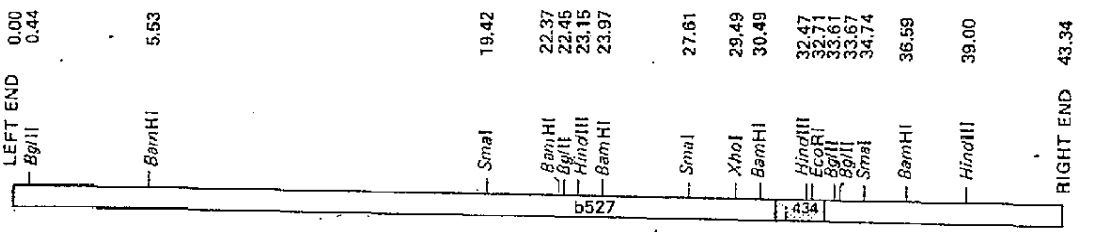

Figure 1. Map of λgt10. Restriction endonuclease cleavage sites are designated in kilobase pairs from the left end. The *b*527 deletion removes DNA sequences between 49.1% and 57.4% on the wild-type λ map. The *imm*^{434} substitution replaces DNA sequences between 72.9% and 79.3%.

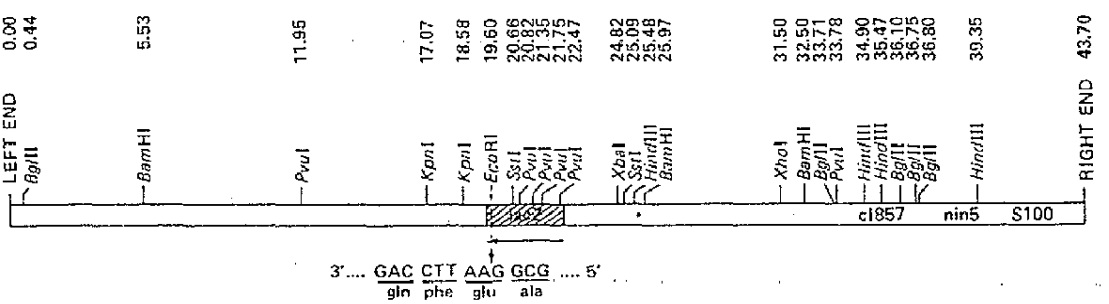

Figure 2. Map of λgt11. Restriction endonuclease cleavage sites are designated in kilobase pairs from the left end. *, λ attachment site. The transcriptional orientation of *lacZ* is indicated by the horizontal arrow. The sequence of the unique *Eco*RI site, the nucleotides that immediately surround it, and the amino acids encoded are shown below the phage map.

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Source: https://www.industrydocuments.ucsf.edu/docs/fxwk0001